
GENE THERAPY - TOOLS AND POTENTIAL APPLICATIONS

Edited by **Francisco Martin Molina**

Gene Therapy - Tools and Potential Applications

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Preface

In the last 10 years gene therapy has experienced a renaissance thanks to the development of safer and more efficient gene transfer vectors and to the advances in the cell therapy field. This book brings together a comprehensive collection of gene therapy tools and their therapeutic applications. The first part of the book covers different gene therapy vectors focusing on their advantages and disadvantages. The second part of the book gets into gene therapy applications, from the latest successes on clinical trials to the new gene therapy targets that are still under development. This book allows the reader to come across with the opinions of different experts in the gene therapy field.

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Introduction

Non-Viral Delivery Systems in Gene Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Recent advances in molecular biology combined with the culmination of the Human Genome Project [1] have provided a genetic understanding of cellular processes and disease pathogenesis; numerous genes involved in disease and cellular processes have been identified as targets for therapeutic approaches. In addition, the development of high-throughput screening techniques (e.g., cDNA microarrays, differential display and database mining) may drastically increase the rate at which these targets are identified [2,3]. Over the past years there has been a remarkable expansion of both the number of human genes directly associated with disease states and the number of vector systems available to express those genes for therapeutic purposes. However, the development of novel therapeutic strategies using these targets is dependent on the ability to manipulate the expression of these target genes in the desired cell population. In this chapter we explain the concept and aim of gene therapy, the different gene delivery systems and therapeutic strategies, how genes are delivered and how they reach the target.

2. Aim and concept of gene therapy with non-viral vectors

A gene therapy medicinal product is a biological product which has the following characteristics: (a) it contains an active substance which contains or consists of a recombinant nucleic acid used in administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence [4].

The most important, and most difficult, challenge in gene therapy is the issue of delivery. The tools used to achieve gene modification are called gene therapy vectors and they are the “key” for an efficient and safe strategy. Therefore, there is a need for a delivery system, which must first overcome the extracellular barriers (such as avoiding particle clearance mechanisms, targeting specific cells or tissues and protecting the nucleic acid from degradation) and, subsequently, the cellular barriers (cellular uptake, endosomal escape, nuclear entry and nucleic release) [5]. An ideal gene delivery vector should be effective, specific, long lasting and safe.

Gene therapy has long been regarded a promising treatment for many diseases, including inherited through a genetic disorder (such as hemophilia, human severe combined immunodeficiency, cystic fibrosis, etc) or acquired (such as AIDS or cancer). Figures 1 and 2 show the indications addressed and the gene types transferred in gene therapy clinical trials, respectively [6].

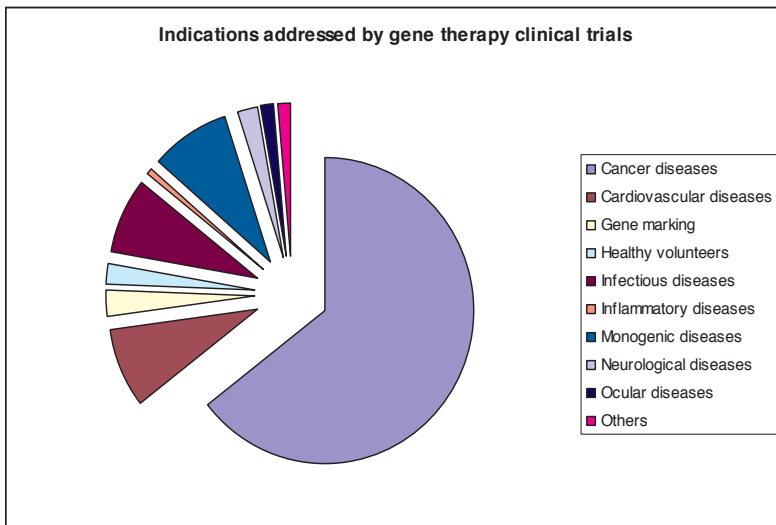


Figure 1. Indications addressed by gene therapy clinical trials (adapted from <http://www.wiley.co.uk/genmed/clinical>).

Gene delivery systems include viral vectors and non-viral vectors. Viral vectors are the most effective, but their application is limited by their immunogenicity, oncogenicity and the small size of the DNA they can transport. Non-viral vectors are safer, of low cost, more reproducible and do not present DNA size limit. The main limitation of non-viral systems is their low transfection efficiency, although it has been improved by different strategies and the efforts are still ongoing [6]; actually, advances of non-viral delivery have lead to an increased number of products entering into clinical trials. However, viral vector has dominated the clinical trials in gene therapy for its relatively high delivery efficiency. Figure 3 shows the proportion of vector systems currently in human trials [7].

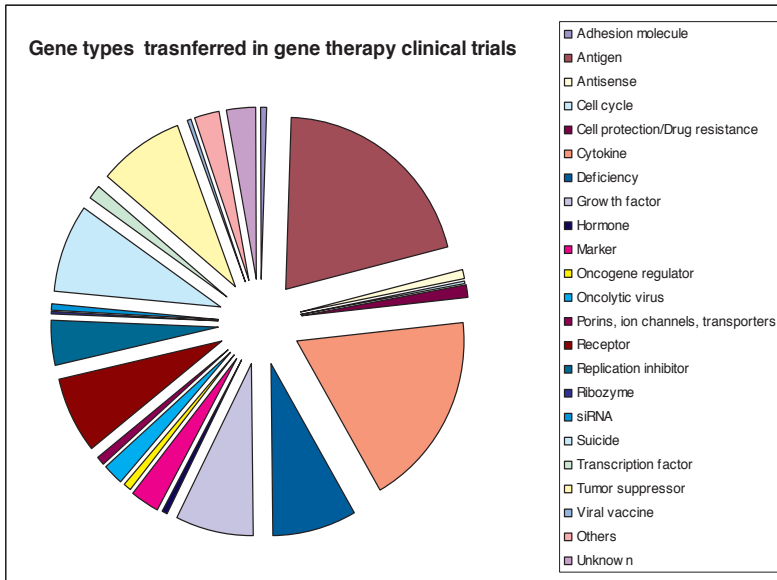


Figure 2. Gene types transferred in gene therapy clinical trials (adapted from <http://www.wiley.co.k/genmed/clinical>).

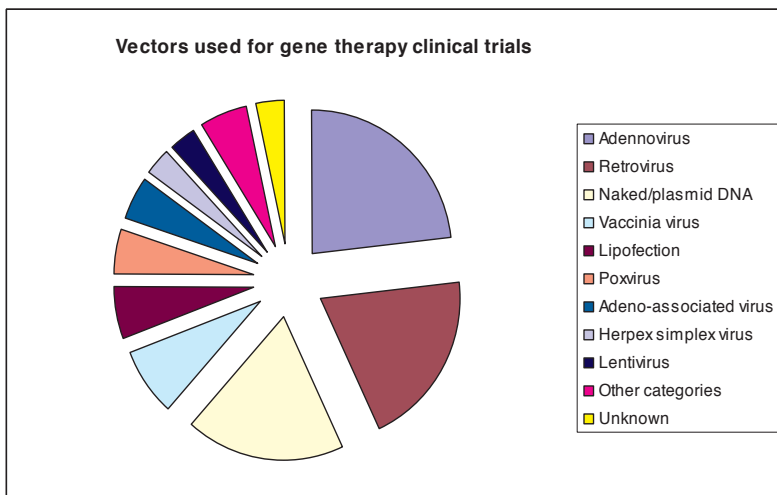


Figure 3. Vector systems used in gene therapy clinical trials (adapted from <http://www.wiley.co.k/genmed/clinical>).

3. Non-viral methods for transfection

Currently, three categories of non-viral systems are available:

- Inorganic particles
- Synthetic or natural biodegradable particles
- Physical methods

Table 1 summarizes the most utilized non-viral vectors.

Category	System for gene delivery
Inorganic particles	Calcium phosphate
	Silica
	Gold
	Magnetic
Synthetic or natural biodegradable particles	1. Polymeric-based non-viral vectors:
	Poly(lactic-co-glycolic acid) (PLGA)
	Poly lactic acid (PLA)
	Poly(ethylene imine) (PEI)
	Chitosan
	Dendrimers
	Polymethacrylates
	2. Cationic lipid-based non-viral vectors:
	Cationic liposomes
	Cationic emulsions
	Solid lipid nanoparticles
Physical methods	3. Peptide-based non-viral vectors:
	Poly-L-lysine
	Other peptides to functionalize other delivery systems: SAP, protamine
	Needle injection
	Balistic DNA injection
	Electroporation
	Sonoporation
	Photoporation
	Magnetofection
	Hydroporation

Table 1. Delivery systems for gene therapy.

3.1. Inorganic particles

Inorganic nanoparticles are nanostructures varying in size, shape and porosity, which can be engineered to evade the reticuloendothelial system or to protect an entrapped molecular payload from degradation or denaturation [8]. Calcium phosphate, silica, gold, and several magnetic compounds are the most studied [9-11]. Silica-coated nanoparticles are biocompatible structures that have been used for various biological applications including gene therapy due to its biocompatibility [8]. Mesoporous silica nanoparticles have shown gene transfection efficiency “in vitro” in glial cells [12]. Magnetic inorganic nanoparticles (such as Fe_3O_4 , MnO_2) have been applied for cancer-targeted delivery of nucleic acids and simultaneous diagnosis via magnetic resonance imaging [13,14]. Silica nanotubes have been also studied as an efficient gene delivery and imaging agent [13].

Inorganic particles can be easily prepared and surface-functionalized. They exhibit good storage stability and are not subject to microbial attack [13]. Bhattarai et al. [15] modified mesoporous silica nanoparticles with poly(ethylene glycol) and methacrylate derivatives and used them to deliver DNA or small interfering RNA (siRNA) “in vitro”.

Gold nanoparticles have been lately investigated for gene therapy. They can be easily prepared, display low toxicity and the surface can be modified using various chemical techniques [16]. For instance, gold nanorods have been proposed to deliver nucleic acids to tumors [13]. They have strong absorption bands in the near-infrared region, and the absorbed light energy is then converted into heat by gold nanorods (photothermal effect). The near-infrared light can penetrate deeply into tissues; therefore, the surface of the gold could be modified with double-stranded DNA for controlled release [17]. After irradiation with near-infrared light, single stranded DNA is released due to thermal denaturation induced by the photo-thermal effect.

3.2. Synthetic or natural biodegradable particles

Synthetic or natural biocompatible particles may be composed by cationic polymers, cationic lipids or cationic peptides, and also the combination of these components [18-21]. The potential advantages of biodegradable carriers are their reduced toxicity (degradation leads to non-toxic products) and avoidance of accumulation of the polymer in the cells.

3.2.1. Polymer-based non-viral vectors

Cationic polymers condense DNA into small particles (polyplexes) and prevent DNA from degradation. Polymeric nanoparticles are the most commonly used type of nano-scale delivery systems. They are mostly spherical particles, in the size range of 1-1000 nm, carrying the nucleic acids of interest. DNA can be entrapped into the polymeric matrix or can be adsorbed or conjugated on the surface of the nanoparticles. Moreover, the degradation of the polymer can be used as a tool to release the plasmid DNA into the cytosol [22]. Table 1 shows several commonly used polymers used for gene delivery [16].

3.2.1.1. *Poly(lactic-co-glycolic acid) (PLGA) and poly lactic acid (PLA)*

Biodegradable polyesters, PLGA and PLA, are the most commonly used polymers for delivering drugs and biomolecules, including nucleic acids. They consist of units of lactic acid and glycolic acid connected through ester linkage. These biodegradable polymers undergo bulk hydrolysis thereby providing sustained delivery of the therapeutic agent. The degradation products, lactic acid and glycolic acid, are removed from the body through citric acid cycle. The release of therapeutic agent from these polymers occurs by diffusion and polymer degradation [16].

PLGA has a demonstrated FDA approved track record as a vehicle for drug and protein delivery [23,24]. Biodegradable PLA and PLGA particles are biocompatible and have the capacity to protect pDNA from nuclease degradation and increase pDNA stability [25,26].

PLGA particles typically less than 10 μm in size are efficiently phagocytosed by professional antigen presenting cells; therefore, they have significant potential for immunization applications [27,28]. For example, intramuscular immunization of p55 Gag plasmid adsorbed on PLGA/cetyl trimethyl ammonium bromide (CTAB) particles induced potent antibody and cytotoxic T lymphocyte responses. These particles showed a 250-fold increase in antibody response at higher DNA doses and more rapid and complete seroconversion, at the lower doses, compared to other adjuvants, including cationic liposomes [29].

The encapsulation efficiency of DNA in PLGA nanoparticles is not very high, and it depends on the molecular weight of the PLGA and on the hydrophobicity of the polymer, being the hydrophilic polymers those that provide higher loading efficiency [30]. To enhance the DNA loading, several strategies have been proposed. Kusunowiriyawong et al. [31] prepared cationic PLGA microparticles by dissolving cationic surfactants (like water insoluble stearylamine) in the organic solvent in which the PLGA was dissolved to prepare the microparticles. Another strategy was to reduce the negative charge of plasmid DNA by condensing it with poly(aminoacids) (like poly-L-lysine) before encapsulation in PLGA microparticles [32,33].

Normally, after an initial burst release, plasmid DNA release from PLGA particles occurs slowly during several days/weeks [22]. The degradation of the PLGA nanoparticles, through a bulk homogeneous hydrolytic process, determines the release of plasmid DNA. Consequently, it can be expected that the use of more hydrophilic PLGA not only improves the encapsulation efficiency of DNA, but also results in a faster release of plasmid DNA. Delivery of the plasmid DNA depends on the copolymer composition of the PLGA (lactic acid versus glycolic acid), molecular weight, particle size and morphology [22]. DNA release kinetics depends also on the plasmid incorporation technique; Pérea et al. [34] reported that nanoparticles prepared by the water-oil emulsion/diffusion technique released their content rapidly, whereas those obtained by the water-oil-emulsion method showed an initial burst followed by a slow release during at least 28 days.

PLGA and PLA based nanoparticles have also been used for “in vitro” RNAi delivery [35]. For instance, Hong et al. [36] have shown the effects of glucocorticoid receptor siRNA deliv-

ered using PLGA microparticles, on proliferation and differentiation capabilities of human mesenchymal stromal cells.

3.2.1.2. Chitosan

Chitosan [b(1-4)2-amino-2-deoxy-D-glucose] is a biodegradable polysaccharide copolymer of N-acetyl-D-glucosamine and D-glucosamine obtained by the alkaline deacetylation of chitin, which is a polysaccharide found in the exoskeleton of crustaceans of marine arthropods and insects [37]. Chitosans differ in the degree of N-acetylation (40 to 98%) and molecular weight (50 to 2000 kDa) [38]. As the only natural polysaccharide with a positive charge, chitosan has the following unique properties as carrier for gene therapy:

- it is potentially safe and non-toxic, both in experimental animals [39] and humans [40]
- it can be degraded into H₂O and CO₂ in the body, which ensures its biosafety
- it has biocompatibility to the human body and does not elicit stimulation of the mucosa and the derma
- its cationic polyelectrolyte nature provides a strong electrostatic interaction with negatively charged DNA [41], and protects the DNA from nuclease degradation [42]
- the mucoadhesive property of chitosan potentially leads to a sustained interaction between the macromolecule being “delivered” and the membrane epithelia, promoting more efficient uptake [43]
- it has the ability to open intercellular tight junctions, facilitating its transport into the cells [44]

Currently, there is a commercial transfection reagent based on chitosan (Novafect, NovaMatrix, FMC, US), and many other prototypes are under development. Most of the chitosan-based nanocarriers for gene delivery have been based on direct complexation of chitosan and the nucleic acid [45], whereas in some instances additional polyelectrolytes, polymers and lipids have been used in order to form composite nanoparticles [46-49] or chitosan-coated hydrophobic nanocarriers.

Many studies using cell cultures have shown that pDNA-loaded chitosan nanocarriers are able to achieve high transfection levels in most cell lines [50]. Chitosan nanocarriers loaded with siRNA have provided gene suppression values similar to the commercial reagent lipofectamine [51,52,18,53].

Chitosan of low molecular weight is more efficient for transfection than chitosan with high molecular weight. This enhancement in transfection efficacy observed with low molecular weight chitosan can be attributed to the easier release of pDNA from the nanocarrier upon cell internalization. Moreover, the presence of free low molecular chitosan has been deemed to be very important for the endosomal escape of the nanocarriers [50]. Concerning deacetylation degree, its influence on transfection is not still clear. “In vitro” studies have shown that the best transfection is achieved with highly deacetylated chitosan [54,55]. However, “in

vivo", higher transfection was achieved after intramuscular administration of chitosan complexes with a low deacetylation degree [55].

3.2.1.3. Poly(ethylene imine) (PEI)

PEI is one of the most potent polymers for gene delivery. PEI is produced by the polymerization of aziridine and has been used to deliver genetic material into various cell types both "in vitro" and "in vivo" [56,57]. There are two forms of this polymer: the linear form and the branched form, being the branched structure more efficient in condensing nucleic acids than the linear PEI [58].

PEI has a high density of protonable amino groups, every third atom being amino nitrogen, which imparts a high buffering ability at practically any pH [16]. Hence, once inside the endosome, PEI disrupts the vacuole releasing the genetic material in the cytoplasm. This ability to escape from the endosome, as well as the ability to form stable complexes with nucleic acids, make this polymer very useful as a gene delivery vector [56].

Depending on the type of polymer (e.g. linear or branched PEI), as well as the molecular weight, the particle sizes of the polyplexes formed are more or less uniformly distributed [59]. Transfection efficiency of PEI has been found to be dependent on a multitude of factors such as molecular weight, degree of branching, N/P ratio, complex size, etc [60].

The use of PEI for gene delivery is limited due to the relatively low transfection efficiency, short duration of gene expression, and elevated toxicity [61,62]. Conjugation of poly(ethylene glycol) to PEI to form diblock or triblock copolymers has been used by some authors to reduce the toxicity of PEI [63,64,65]. Poly(ethylene glycol) also shields the positive charge of the polyplexes, thereby providing steric stability to the complex. Such stabilization prevents non-specific interaction with blood components during systemic delivery [66].

3.2.1.4. Dendrimers

Dendrimers are polymer-based molecules with a symmetrical structure in precise size and shapes, as well as terminal group functionality [8]. Dendrimers contain three regions: i) a central core (a single atom or a group of atoms having two or more identical chemical functionalities); ii) branches emanating from core, which are composed of repeating units with at least one branching junction, whose repetition is organized in a geometric progression that results in a series of radially concentric layers; and iii) terminal function groups. Dendrimers bind to genetic material when peripheral groups, that are positively-charged at physiological pH, interact with the negatively-charged phosphate groups of the nucleic acid [67,68]. Due to their nanometric size, dendrimers can interact effectively and specifically with cell components such as membranes, organelles, and proteins [69].

For instance, Qi et al. [70] showed the ability of generations 5 and 6 (G5 and G6) of poly(amidoamine) (PAMAM) dendrimers, conjugated with poly(ethylene glycol) to efficiently transfect both "in vitro" and "in vivo" after intramuscular administration to neonatal mice. PAMAM has also the ability to deliver siRNAs, especially "in vitro" in cell culture sys-

tems [71-73]. Recent studies showed that the dendrimer-mediated siRNA delivery and gene silencing depends on the stoichiometry, concentration of siRNA and the dendrimer generation [71]. In a recent study, a PAMAM dendrimer-delivered short hairpin RNA (shRNA) showed the ability to deplete a human telomerase reverse transcriptase, the catalytic subunit of telomerase complex, resulting in partial cellular apoptosis, and inhibition of tumor outgrowth in xenotransplanted mice [74].

The toxicity profile of dendrimers is good, although it depends on the number of terminal amino groups and positive charge density. Moreover, toxicity is concentration and generation dependent with higher generations being more toxic as the number of surface groups doubles with increasing generation number [75,76].

3.2.1.5. *Polymethacrylates*

Polymethacrylates are cationic vinyl-based polymers that possess the ability to condense polynucleotides into nanometer size particles. They efficiently condense DNA by forming inter-polyelectrolyte complexes. A range of polymethacrylates, differing in molecular weights and structures, have been evaluated for their potential as gene delivery vector, such as poly[2-dimethylamino) ethyl methacrylate] (DMAEMA) and its co-polymers [16]. The use of polymethacrylates for DNA transfection is, however, limited due to their low ability to interact with membranes.

In order to optimise the use of these compounds for gene transfer, Christiaens et al. [77] combined polymethacrylates with penetratin, a 16-residue water-soluble peptide that internalises into cells through membrane translocation. Penetratin mainly enhanced the endolysosomal escape of the polymethacrylate–DNA complexes and increased their cellular uptake using COS-1 (kidney cells of the African green monkey). Nanoparticles with a methacrylate core and PEI shell prepared via graft copolymerization have also been employed lately for gene delivery [78,79]. This conjugation resulted in nanoparticles with a higher transfection efficiency and lower toxicity as compared with PEI.

3.2.2. *Cationic-lipid based non-viral vectors*

Cationic lipids have been among the more efficient synthetic gene delivery reagents “in vitro” since the landmark publications in the late 1980s [80]. Cationic lipids can condense nucleic acids into cationic particles when the components are mixed together. This cationic lipid/nucleic acid complex (lipoplex) can protect nucleic acids from enzymatic degradation and deliver the nucleic acids into cells by interacting with the negatively charged cell membrane [81]. Lipoplexes are not an ordered DNA phase surrounded by a lipid bilayer; rather, they are a partially condensed DNA complex with an ordered substructure and an irregular morphology [82,83]. Since the initial studies, hundreds of cationic lipids have been synthesized as candidates for non-viral gene delivery [84] and a few made it to clinical trials [85,86].

Cationic lipids can be used to form lipoplexes by directly mixing the positively charged lipids at the physiological pH with the negatively charged DNA. However, cationic lipids are

more frequently used to prepare lipoplex structures such as liposomes, nanoemulsions or solid lipid nanoparticles [81].

3.2.2.1. *Cationic liposomes*

Liposomes are spherical vesicles made of phospholipids used to deliver drugs or genes. They can range in size from 20 nm to a few microns. Cationic liposomes and DNA interact spontaneously to form complexes with 100% loading efficiency; in other words, all of the DNA molecules are complexed with the liposomes, if enough cationic liposomes are available. It is believed that the negative charges of the DNA interact with the positively charged groups of the liposomes [87]. The lipid to DNA ratio, and overall lipid concentration used in forming these complexes, are very important for efficient gene delivery and vary with applications [88].

Liposomes offer several advantages for gene delivery [87]:

- they are relatively cheap to produce and do not cause diseases
- protection of the DNA from degradation, mainly due to nucleases
- they can transport large pieces of DNA
- they can be targeted to specific cells or tissues

Successful delivery of DNA and RNA to a variety of cell types has been reported, including tumour, airway epithelial cells, endothelial cells, hepatocytes, muscle cells and others, by intratissue or intravenous injection into animals [89,90].

Several liposome-based vectors have been assayed in a number of clinical trials for cancer treatment. For instance, Allovectin-7® (Vical, San Diego, CA, USA), a plasmid DNA carrying HLAB and β 2-microglobulin genes complexed with DMRIE/DOPE liposomes have been assessed for safety and efficacy in phase I and II clinical trials [91,92].

3.2.2.2. *Lipid nanoemulsions*

An emulsion is a dispersion of one immiscible liquid in another stabilized by a third component, the emulsifying agent [93]. The nanoemulsion consists of oil, water and surfactants, and presents a droplet size distribution of around 200 nm. Lipid-based carrier systems represent drug vehicles composed of physiological lipids, such as cholesterol, cholesterol esters, phospholipids and tryglicerides, and offer a number of advantages, making them an ideal drug delivery carrier [94]. Adding cationic lipids as surfactants to these dispersed systems makes them suitable for gene delivery. The presence of cationic surfactants, like DOTAP, DOTMA or DC-Chol, causes the formation of positively charged droplets that promote strong electrostatic interactions between emulsion and the anionic nucleic acid phosphate groups [95,96]. For instance, Bruxel et al. [97] prepared a cationic nanoemulsion with DOTAP as a delivery system for oligonucleotides targeting malarial topoisomerase II.

Lipid emulsions are considered to be superior to liposomes mainly in a scaling-up point of view. On the one hand, emulsions can be produced on an industrial scale; on the other

hand, emulsions are stable during storage and are highly biocompatible [94]. In addition, the physical characteristics and serum-resistant properties of the DNA/nanoemulsion complexes suggest that cationic nanoemulsions could be a more efficient carrier system for gene and/or immunogene delivery than liposomes. One of the reasons for the serum-resistant properties of the cationic lipid nanoemulsions may be the stability of the nanoemulsion/DNA complex [98]. However, in spite of extensive research on emulsions, very few reports using cationic amino-based nanoemulsions in gene delivery have been published. After “in vivo” administration, cationic nanoemulsions have shown higher transfection and lower toxicity than liposomes [99].

The incorporation of nonionic surfactant with a branched poly(ethylene glycol), such as Tween 80®, increments the stability of the nanoemulsion and prevent the formation of large nanoemulsion/DNA complexes, probably because of their steric hindrance and the generation of a hydrophilic surface that may enhance the stability by preventing physical aggregation [94]. In addition, this strategy may prevent from enzymatic degradation in blood, and due to the hydrophilic surface, they are taken up slowly by phagocytic cells, resulting in prolonged circulation in blood [100,101].

3.2.2.3. *Solid lipid nanoparticles (SLN)*

Solid lipid nanoparticles are particles made from a lipid being solid at room temperature and also at body temperature. They combine advantages of different colloidal systems. Like emulsions or liposomes, they are physiologically compatible and, like polymeric nanoparticles, it is possible to modulate drug release from the lipid matrix. In addition, SLN possess certain advantages. They can be produced without use of organic solvents, using high pressure homogenization (HPH) method that is already successfully implemented in pharmaceutical industry [102]. From the point of view of application, SLN have very good stability [103] and are subject to be lyophilized [104], which facilitates the industrial production.

Cationic SLN, for instance, SLN containing at least one cationic lipid, have been proposed as non-viral vectors for gene delivery [105,20]. It has been shown that cationic SLN can effectively bind nucleic acids, protect them from DNase I degradation and deliver them into living cells. Cationic lipids are used in the preparation of SLN applied in gene therapy not only due to their positive surface charge, but also due to their surfactant activity, necessary to produce an initial emulsion, which is a common step in most preparation techniques. By means of electrostatic interactions, cationic SLN condense nucleic acids on their surface, leading generally to an excess of positive charges in the final complexes. This is beneficial for transfection because condensation facilitates the mobility of nucleic acids, protects them from environmental enzymes and the cationic character of the vectors allows the interaction with negatively charged cell surface. The characteristics of the resulting complexes depend on the ratio between particle and nucleic acid; there must be an equilibrium between the binding forces of the nucleic acids to SLN to achieve protection without hampering the posterior release in the site of action [106]. Release of DNA from the complexes may be one of the most crucial steps determining the optimal ratio for cationic lipid system-mediated transfection [107].

Our research group showed for the first time the expression of a foreign protein with SLNs in an “in vivo” study [108]. After intravenous administration of SLN containing the EGFP plasmid to BALB/c mice, protein expression was detected in the liver and spleen from the third day after administration, and it was maintained for at least 1 week. In a later study [109], we incorporated dextran and protamine in the SLN and the transfection was improved, being detected also in lung. The improvement in the transfection was related to a longer circulation in the bloodstream due to the presence of dextran on the nanoparticle surface. The surface features of this new vector may also induce a lower opsonization and a slower uptake by the RES. Moreover, the high DNA condensation of protamine that contributes to the nuclease resistance may result in an extended stay of plasmid in the organism. The presence of nuclear localization signals in protamine, which improves the nuclear envelope translocation, and its capacity to facilitate transcription [110] may also explain the improvement of the transfection efficacy “in vivo”.

SLN have also been applied for the treatment of ocular diseases by gene therapy. After ocular injection of a SLN based vector to rat eyes, the expression of EGFP was detected in various types of cells depending on the administration route: intravitreal or subretinal. In addition, this vector was also able to transfect corneal cells after topical application [111].

SLN may also be used as delivery systems for siRNA or oligonucleotides. Apolipoprotein-free low-density lipoprotein (LDL) mimicking SLN [112] formed stable complexes with siRNA and exhibited comparable gene silencing efficiency to siRNA complexed with the polymer PEI, and lower cytotoxicity. Afterwards, Tao et al. [113] showed that lipid nanoparticles caused 90% reduction of luciferase expression for at least 10 days, after a single systemic administration of 3 mg/kg luciferase siRNA to a liver-luciferase mouse model. CTAB stabilized SLN bearing an antisense oligonucleotide against glucosylceramide synthase (asGCS) reduced the viability of the drug resistant NCI/ADR-RES human ovary cancer cells in the presence of the chemotherapeutic doxorubicin [114].

3.2.3. *Peptide-based gene non-viral vectors*

Many types of peptides, which are generally cationic in nature and able to interact with plasmid DNA through electrostatic interaction, are under intense investigation as a safe alternative for gene therapy [115]. There are mainly four barriers that must be overcome by non-viral vectors to achieve successful gene delivery. The vector must be able to tightly compact and protect DNA, target specific cell-surface receptors, disrupt the endosomal membrane, and deliver the DNA cargo to the nucleus [115]. Peptide-based vectors are advantageous over other non-viral systems because they are able to achieve all of these goals [116]. Cationic peptides rich in basic residues such as lysine and/or arginine are able to efficiently condense DNA into small, compact particles that can be stabilized in serum [117,118]. Attachment of a peptide ligand to a polyplex or lipoplex allows targeting to specific receptors and/or specific cell types. Peptide sequence derived from protein transduction domains are able to selectively lyse the endosomal membrane in its acidic environment leading to cytoplasmic release of the particle [119,120]. Finally, short peptide sequences taken

from longer viral proteins can provide nuclear localization signals that help the transport of the nucleic acids to the nucleus [121,122].

3.2.3.1. *Poly-L-lysine*

Poly-L-lysine is a biodegradable peptide synthesized by polymerization on N-carboxy-anhydride of lysine [123]. It is able to form nanometer size complexes with polynucleotides owing to the presence of protonable amine groups on the lysine moiety [16]. The most commonly used poly-L-lysine has a polymerization degree of 90 to 450 [124]. This characteristic makes this peptide suitable for “in vivo” use because it is readily biodegradable [116]. However, as the length of the poly-L-lysine increases, so does the cytotoxicity. Moreover, poly-L-lysine exhibits modest transfection when used alone and requires the addition of an endosomolytic agent such as chloroquine or a fusogenic peptide to allow for release into the cytoplasm. An strategy to prevent plasma protein binding and increase circulation half-life is the attachment of poly(ethylene glycol) to the poly-L-lysine [125,126].

3.2.3.2. *Peptides in multifunctional delivery systems*

Due to the advantages of peptides for gene delivery, they are frequently used to functionalize cationic lipoplexes or polyplexes. Since these vectors undergo endocytosis, decorating them with endosomolytic peptides for enhanced cytosolic release may be helpful. Moreover, combination with peptides endowed with the ability to target a specific tissue of interest is highly beneficial, since this allows for reduced dose and, therefore, reduced side effects following systemic administration [127]. In a study carried out by our group [19], we improved cell transfection of ARPE-19 cells by using a cell penetration peptide (SAP) with solid lipid nanoparticles. Kwon et al. [128] covalently attached a truncated endosomolytic peptide derived from the carboxy-terminus of the HIV cell entry protein gp41 to a PEI scaffold, obtaining improved gene transfection results compared with unmodified PEI. In other study [20], protamine induced a 6-fold increase in the transfection capacity of SLN in retinal cells due to a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis, which promotes the release of the protamine-DNA complexes from the solid lipid nanoparticles; afterwards the transport of the complexes into the nucleus is favoured by the nuclear localization signals of the protamine.

3.3. Physical methods for gene delivery

Gene delivery using physical principles has attracted increasing attention. These methods usually employ a physical force to overcome the membrane barrier of the cells and facilitate intracellular gene transfer. The simplicity is one of the characteristics of these methods. The genetic material is introduced into cells without formulating in any particulate or viral system. In a recent publication, Kamimura et al. [87] revised the different physical methods for gene delivery. These methods include the following:

3.3.1. *Needle injection*

The DNA is directly injected through a needle-carrying syringe into tissues. Several tissues have been transfected by this method [87]: muscle, skin, liver, cardiac muscle, and solid tumors. DNA vaccination is the major application of this gene delivery system [129]. The efficiency of needle injection of DNA is low; moreover, transfection is limited to the needle surroundings.

3.3.2. *Ballistic DNA injection*

This method is also called particle bombardment, microprojectile gene transfer or gene gun. DNA-coated gold particles are propelled against cells, forcing intracellular DNA transfer. The accelerating force for DNA-containing particles can be high-voltage electronic discharge, spark discharge or helium pressure discharge. One advantage of this method is that it allows delivering precise DNA doses. However, genes express transiently, and considerable cell damage occurs at the centre of the discharge site. This method has been used in vaccination against the influenza virus [130] and in gene therapy for treatment of ovarian cancer [131].

3.3.3. *Electroporation*

Gene delivery is achieved by generating pores on a cell membrane through electric pulses. The efficiency is determined by the intensity of the pulses, frequency and duration [132]. Electroporation creates transient permeability of the cell membrane and induces a low level of inflammation at the injection site, facilitating DNA uptake by parenchyma cells and antigen-presenting cells [133]. As drawbacks, the number of cells transfected is low, and surgery is required to reach internal organs. This method has been clinically tested for DNA-based vaccination [134] and for cancer treatment [135].

3.3.4. *Sonoporation*

Sonoporation utilizes ultrasound to temporally permeabilize the cell membrane to allow cellular uptake of DNA. It is non-invasive and site-specific and could make it possible to destroy tumor cells after systemic delivery, while leave non-targeted organs unaffected [13]. Gene delivery efficiency seems to be dependent on the intensity of the pulses, frequency and duration [87]. This method has been applied in the brain, cornea, kidney, peritoneal cavity, muscle, and heart, among others. Low-intensity ultrasound in combination with microbubbles has recently acquired much attention as a safe method of gene delivery [13]. The use of microbubbles as gene vectors is based on the hypothesis that destruction of DNA-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will result in localized transduction upon disruption of the microbubble shell while sparing non-targeted areas. The therapeutic effect of ultrasound-targeted microbubble destruction is relative to the size, stability, and targeting function of microbubbles.

3.3.5. Photoporation

The photoporation method utilizes a single laser pulse as the physical force to generate transient pores on a cell membrane to allow DNA to enter [87]. Efficiency seems to be controlled by the size of the focal point and pulse frequency of the laser. The level of transgene expression reported is similar to that of electroporation. Further studies are needed before this highly sophisticated procedure becomes a practical technique for gene delivery.

3.3.6. Magnetofection

This method employs a magnetic field to promote transfection. DNA is complexed with magnetic nanoparticles made of iron oxide and coated with cationic lipids or polymers through electrostatic interaction. The magnetic particles are then concentrated on the target cells by the influence of an external magnetic field. Similar to the mechanism of non-viral vector-based gene delivery, the cellular uptake of DNA is due to endocytosis and pinocytosis [136]. This method has been successfully applied to a wide range of primary cells, and cells that are difficult to transfect by other non-viral vectors [137].

3.3.7. Hydroporation

Hydroporation, also called hydrodynamic gene delivery method, is the most commonly method used for gene delivery to hepatocytes in rodents. Intrahepatic gene delivery is achieved by a rapid injection of a large volume of DNA solution via the tail vein in rodents, that results in a transient enlargement of fenestrae, generation of a transient membrane defect on the plasma membrane and gene transfer to hepatocytes [87]. This method has been frequently employed in gene therapy research. In order to apply this simple method of gene administration to the clinic, efforts have been made to reduce the injection volume and avoid tissue damage.

4. Strategies to improve transfection mediated by non-viral vectors

The successful delivery of therapeutic genes to the desired target cells and their availability at the intracellular site of action are crucial requirements for efficient gene therapy. The design of safe and efficient non-viral vectors depends mainly on our understanding of the mechanisms involved in the cellular uptake and intracellular disposition of the therapeutic genes as well as their carriers. Moreover, they have to overcome the difficulties after “in vivo” administration.

4.1. Target cell uptake and intracellular trafficking

Nucleic acid must be internalized to interact with the intracellular machinery to execute their effect. The positive surface charge of unshielded complexes facilitates cellular internalization. The non-viral vector can be functionalized with compounds that are recognized by the desired specific target cell type. Peptides, proteins, carbohydrates and small molecules

have been used to induce target cell-specific internalization [138]. For instance, SLN have been combined with peptides that show penetrating properties, such as the dimeric HIV-1 TAT (Trans-Activator of Transcription) peptide [139] or the synthetic SAP (Sweet Arrow Peptide) [19].

Endocytosis has been postulated as the main entry mechanism for non-viral systems. Various endocytosis mechanisms have been described to date: phagocytosis, pinocytosis, clathrin-mediated endocytosis, caveolae/raft-mediated endocytosis and chathrin and caveolae independent endocytosis. Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes, which degrade their content, whereas caveolae/raft-mediated endocytosis avoids the lysosomal pathway and its consequent vector degradation [20]. Cytosolic delivery from either endosomes or lisosomes has been reported a major limitation in transfection [140]. In consequence, some research groups have used substances that facilitate endosomal escape before lysosomal degradation. For clathrin-mediated endocytosis, the drop in pH is a useful strategy for endosomal scape via proton destabilization conferred by the cationic carrier, or by pH-dependent activation of membrane disruptive helper molecules, such as DOPE or fusogenic peptides [141-143]. More recently, Leung et al. [144] have patented lipids with 4-amino-butyric acid (FAB) as headgroup to form lipid nanoparticles able to introduce nucleic acids, specifically siRNA, into mammalian cells. FAB lipids also demonstrated membrane destabilizing properties.

Once genes are delivered in the cytoplasm they have to diffuse toward the nuclear region. DNA plasmids have difficulties to diffuse in the cytoplasm because they are large in size. Therefore, packaging and complexing them into small particles facilitates its displacement intracellularly. Diffusion is a function of diameter; hence, smaller particles move faster than larger ones. Thus, another way to optimize gene delivery to the nucleus would be to decrease the size of the particles to increase the velocity of passive diffusion through the cytoplasm [145].

The pass through the nuclear membrane is the next step, and it is in general, quite difficult. There are two mechanisms large molecules can use to overcome that barrier: disruption of the nuclear membrane during mitosis, which is conditioned by the division rate of targeted cells, or import through the nuclear pore complex (NPC). This latter mechanism requires nuclear localization signals, which can be used to improve transfection by non-viral vectors [146]. In this regard, protamine is a peptide that condenses DNA and presents sequences of 6 consecutive arginine residues [147], which make this peptide able to translocate molecules such as DNA from the cytoplasm to the nucleus of living cells. Although protamine/DNA polyplexes are not effective gene vectors [148], the combination of protamine with SLN produced good results in both COS-1 and Na 1330 (murine neuroblastoma) culture cells [149,150]. Precondensation of plasmids with this peptide, to form protamine-DNA complexes that are later bound to cationic SLN, is another alternative that has shown higher transfection capacity in retinal cells compared to SLN prepared without protamine [20].

Once inside the nucleus, level of transgene expression depends on the copy number of DNA and its accessibility for the transcription machinery. Studies have shown that the minimum number of plasmids delivered to the nucleus required for measurable transgene expression

depends on the type of vectors [145]. Comparisons between different delivery vehicles showed that higher copy numbers of DNA molecules in the nucleus do not necessarily correlate with higher transfection efficiency. At similar plasmid/nucleus copies, lipofectamine mediated 10-fold higher transfection efficiency than PEI. This suggests that the DNA delivered by PEI is biologically less active than the DNA delivered by lipofectamine. It also emphasizes that a deeper understanding of the nuclear events in gene delivery is required for future progress.

4.2. “In vivo” optimization

Vectors mediating high transfection efficiency “in vitro” often fail to achieve similar results “in vivo”. One possible reason is that lipidic and polymeric vectors are optimized “in vitro” using two-dimensional (2D) cultures that lack extracellular “in vivo” barriers and do not realistically reflect “in vivo” conditions. While cells “in vitro” grow in monolayers, cells “in vivo” grow in 3D tissue layers held together by the extracellular matrix [145]. This results in cells with reduced thicknesses but larger widths and lengths. Particles that are taken up directly above the nucleus (supranuclear region) have the shortest transport distance to the nucleus and hence a greater chance of delivery success. The spatiotemporal distribution of carriers, however, determines the optimal time for endosomal escape and the optimal intracellular pathway [151]. This highlights the need to develop adequate “in vitro” models that mimics as much as possible the “in vivo” conditions to optimize carriers for gene therapy.

After intravenous administration, plasma nuclease degradation of the nucleic acid is the first barrier that needs to be overcome for therapeutic nucleic acid action. Nucleic acids can be degraded by hydrolytic endo- and exo-nucleases. Both types of nucleases are present in blood. Therefore, increasing nuclease resistance is crucial for achieving therapeutic effects. Naked nucleic acids are not only rapidly degraded upon intravenous injection, they are also cleared from the circulation rapidly, further limiting target tissue localization [138]. To improve nuclease resistance and colloidal stability, complexation strength is an important factor. Shielding the non-viral vectors with poly-L-lysine or poly(ethylene glycol), as mentioned previously, prolongs the circulation time in blood of the vectors.

Vectors delivered “in vivo” by systemic administration not only have to withstand the bloodstream, but also have to overcome the cellular matrix to reach all cell layers of the tissue. While large particles seem to have an advantage “in vitro” due to a sedimentation effect on cells, efficient delivery of particles deep into organs requires particles <100 nm. Small particles (40 nm) diffuse faster and more effectively in the extracellular matrix and inner layers of tissues, whereas larger particles (>100 nm) are restricted by steric hindrance [152].

The net cationic charge of the synthetic vector is a determinant of circulation time, tissue distribution and cellular uptake of synthetic vectors by inducing interactions with negatively charged blood constituents, such as erythrocytes and proteins. The opsonisation of foreign particles by plasma proteins actually represents one of the steps in the natural process of removal of foreign particles by the innate immune system [153]. This may result in obstruction of small capillaries, possibly leading to serious complication, such as pulmonary embolism [154]. Part of the complexes end up in the reticuloendothelial system (RES), where they are re-

moved rapidly by phagocytosis or by trapping in fine capillary beds [155]. The nanocarriers, when circulating in blood, can activate the complement system and it seems that the complement activation is higher as the surface charge increases [156,157].

The interaction with blood components is related to the intrinsic properties of the cationic compound (side chain end groups, its spatial conformation and molecular weight), as well as the applied Nitrogen:Phosphate (N:P) ratio [138]. Shielding of the positive surface charge of complexes is currently an important strategy to circumvent the aforementioned problems. The most popular strategy is based on the attachment of water-soluble, neutral, flexible polymers, as poly(ethylene glycol), poly(vinylpyrrolidone) and poly(hydroxyethyl-L-asparagine). The efficacy of the shielding effect of these polymers is determined by the molecular weight and grafting density of the shielding polymer [158]. Longer chains are usually more effective in protecting the particle (surface) from aggregation and opsonisation.

The nanocarriers must arrive to the target tissue to exert their action. Although most commonly used targeting strategies consist of proteins and peptides, carbohydrates have also been utilized [159]. The access of non-viral vector to tumors has been investigated extensively. The discontinuous endothelial cell layer has gaps that give the nanocarriers the opportunity to escape the vascular bed and migrate into the tumoral mass. The most common entities used for tumor targeting include transferrin, epidermal growth factor, and the integrin-binding tripeptide arginine-glycine-aspartic acid (RGD) [159]. Brain targeting has also a great interest; most gene vector do not cross the blood-brain barrier (BBB) after intravenous administration and must be administered through intracerebral injection, which is highly invasive and does not allow for delivery of the gene to other areas of the brain. Injection in the cerebrospinal fluid is also another strategy. Commonly used ligands for mediated uptake are insuline-like growth factors, transferrin or low-density lipid protein [159]. Targeting to the liver has been also investigated in a great extension by many researchers. Carbohydrate-related molecules, such as galactose, asialofetuin, N-acetylgalactosamine and folic acid are the most commonly molecules used for liver targeting [159]. Targeting to endothelial cells provides avenues for improvement of specificity and effectiveness of treatment of many diseases, such as cardiovascular or metabolic diseases [160]. Among other endothelial cell surface determinants, intercellular adhesion molecule-1 (CD54 or ICA-1, a 110-KDa Ig-like transmembrane constitutive endothelial adhesion molecule) is a good candidate target for this goal. ICAM-1 targeting can be achieved by coupling Anti-ICAM-1 antibodies to carriers [161].

5. Conclusion

The success of gene therapy is highly dependent on the delivery vector. Viral vectors have dominated the clinical trials in gene therapy for its relatively high delivery efficiency. However, the improvement of efficacy of non-viral vectors has lead to an increased number of products entering into clinical trials. A better understanding of the mechanisms governing the efficiency of transfection, from the formation of the complexes to their intracellular delivery, will lead to the design of better adapted non-viral vectors for gene therapy applica-

tions. A number of potentially rate-limiting steps in the processes of non-viral-mediated gene delivery have been identified, which include the efficiency of cell surface association, internalization, release of gene from intracellular compartments such as endosomes, transfer via the cytosol and translocation into the nucleus and transcription efficacy. Insight into molecular features of each of these steps is essential in order to determine their effectiveness as a barrier and to identify means of overcoming these hurdles. Although non-viral vectors may work reasonably well “in vitro”, clinical success is still far from ideal. Considering the number of research groups that focus their investigations on the development of new vectors for gene therapy, together with the advances in the development of new technologies to better understand their “in vitro” and “in vivo” behavior, the present limitations of non-viral vectors will be resolved rationally.

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Plasmid Transgene Expression *in vivo*: Promoter and Tissue Variables

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Additional information is available at the end of the chapter

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1. Introduction

Ensuring an appropriate level and duration of expression is essential in achieving an efficient and safe gene therapy. While the length of time a gene must be expressed for efficacy depends on both the therapeutic strategy and the disease, many gene therapy approaches prove ineffective as the therapeutic is expressed for a limited duration (Frank et al. 2004). Proposed causes of transient expression include loss of DNA due to cell turnover, immune responses against transfected cells and/or expressed proteins, and inhibition of transcription through host cell methylation of microbial DNA sequences (Prosch et al. 1996; Scheule 2000; Greenland et al. 2007). Vector related elements or activity also contribute to duration of gene expression post administration. Adenovirus is known to stimulate severe innate and adaptive immune responses, and can induce cellular and humoral responses to the transgene product and its capsid proteins resulting in failure to provide long-term gene expression (Jooss et al. 1998; Yuasa et al. 2002; Louboutin et al. 2005; Wang et al. 2005).

Plasmid electroporation, on the other hand, has been shown not to elicit such transgene gene silencing immune responses (Jooss et al. 1998; Mir et al. 1999), and presents an attractive option in achieving long-term gene expression, especially in light of recent improvements in plasmid vectors (Gill et al. 2009). Although plasmid based systems offer certain advantages, they do, however, have drawbacks. The magnitude of transgene expression is generally lower with plasmid vectors than that with viruses. In addition, most plasmids are not passed on to daughter cells following cell division leading to eventual loss of expression in rapidly dividing tissues. This can result in sub-therapeutic effects, a significant problem

with gene therapy. Efforts have been made to ensure that therapeutic protein production is active for an appropriate length of time to address some of these failings. To counteract the effects of episomal DNA loss, the use of integrating DNA in the form of retroviruses or transposon containing plasmids has been examined and shown some efficacy (Sandrin et al. 2003; Ohlfest et al. 2005). Delivery in this fashion would lead to long lasting, possibly indefinite gene expression. Although this addresses one failing of plasmid delivery, the potential of indefinite and uncontrollable protein production to cause unexpected side effects is an issue. Unlike the current situation, where therapy related complications results in withdrawal of the medication, the “offending gene” cannot easily be removed, and may continue to cause significant side effects. In addition, integration of foreign DNA is not ideal as it can lead to mutagenesis, with subsequent alteration in the patient’s protein expression profile and potentially carcinogenesis. With this in mind, methods of prolonging and/or controlling episomal gene expression are preferred, provided this expression is of sufficient magnitude.

Plasmid loss alone may not fully account for the temporal loss of expression seen with these vectors. Epigenetic modification of the therapeutic has also been implicated in gene silencing, but the exact mechanisms by which this occurs have not yet been fully elucidated. It has been demonstrated that duration of transgene expression may be increased by use of ‘native’ promoters of mammalian origin rather than viral promoters (Gazdhar et al. 2006). The postulated mechanism behind this difference of expression relates to the presence and subsequent methylation of CpG sequences on promoters. This methylation is a naturally occurring phenomenon and reports have correlated methylation of CpG-rich sequences with silencing of gene expression (Gazdhar, Bilici et al. 2006). Native mammalian promoters possess fewer CpG sequences than their viral counterparts and are theoretically less prone to silencing. By employing mammalian promoters, the duration of gene expression may be extended, allowing for sustained therapeutic production. Anecdotal evidence suggests that the degree of viral promoter silencing varies between tissue types, and that the duration of gene expression in tumour tissue in particular may be short-lived (Jaenisch et al. 1985; Momparler & Bovenzi 2000; Bartoli et al. 2003). This may, in part, be due to abnormal cell turnover in tumour tissue, but the disorganised methylation pattern in tumour tissue could also play a role.

In this chapter, we assess the influence of promoter type on electroporated plasmid transgene expression in murine models. Expression is examined by utilising the reporter gene luciferase. The activity of luciferase can then be measured *in vivo*, allowing for repeated assessment of gene expression in the same test subjects over time. The pattern of expression is also examined in different tissue types as is the role of epigenetic modification in gene silencing.

2. Materials and methods

2.1. DNA constructs

pGL3-Control and pCMV-luc were purchased from Stratagene (Techno-Path, Limerick, Ireland) and Promega (Medical Supply Co., Dublin, Ireland) respectively. pDRIVE03-UbiquitinB(h) v02 was purchased from Invivogen (Cayla SAS, Toulouse, France). A version of this

plasmid, designated pUb-luc, containing the firefly *luciferase* gene transcriptionally controlled from the human Ubiquitin-B promoter was constructed, by excising the firefly *luciferase* gene from pGL3-Control using restriction enzymes Nco1 and Xba1 (New England Biolabs, USA) and cloning it in the Nhe1 (site 2) and Nco1 sites of pDRIVE03-UbiquitinB(h) downstream of the ubiquitin promoter. Plasmid copy number was calculated using the formula number DNA copies = weight/(Plasmid size \times 1.096×10^{21}) with pUb-luc = 4.3×10^3 , pCMV-luc = 5.9×10^3 and pGL3 = 5.2×10^3 bp respectively. Endotoxin-free plasmid DNA was isolated from TOP10F *E.coli* (Invitrogen) using the MegaPrep kit (Qiagen, West Sussex, England).

2.2. Animals and tumour induction

Murine JBS fibrosarcoma tumour cells were maintained in culture in Dulbecco's Modified Essential Medium (DMEM) (GIBCO, Invitrogen Corp., Paisley, Scotland) as previously described (Collins, C. G. et al. 2006; Collins, S. A. et al. 2010). Female Balb/C and MF1nu/nu mice of 6–8 weeks of age were obtained from Harlan Laboratories (Oxfordshire, England). For routine tumour induction, 2×10^6 JBS cells suspended in 200 μ l serum free DMEM were injected subcutaneously into the flank.

2.3. *In-vivo* gene delivery

For tumour experiments, mice were treated at a tumour volume of approximately 100 mm³ in volume (5–7 mm major diameter). Mice were anaesthetized during all treatments by intraperitoneal (i.p.) administration of 200 μ g xylazine and 2 mg ketamine. For liver transfection, a 1 cm subcostal incision was made over the liver and the peritoneum opened. The right lobe of the exposed liver was administered plasmid by electroporation as described below (Casey et al. 2010; Collins, S. A. et al. 2011). The wound was closed in two layers, peritoneal and skin, using 4/0 prolene sutures (Promed, Killorglin, Ireland). For plasmid delivery by electroporation, a custom-designed applicator with 2 needles 4 mm apart was used, with both needles placed through the skin central to the tissue. Tissue was injected between electrode needles with 8×10^{11} copies of plasmid DNA in sterile injectable saline in an injection volume of 50 μ l. After 80 seconds, square-wave pulses (1200 V/cm 100 μ sec \times 1 and 120 V/cm 20 msec, 8 pulses) were administered in sequence using a custom designed pulse generator (Cliniporator (IGEA, Carpi, Italy)).

2.4. Inhibition of DNA acetylation *in vivo*

Individual animals were weighed and dosed by i.p. injection of trichostatin A (TSA) (Sigma) at 10 mg/kg in 60 μ l 10% (v/v) dimethyl sulfoxide in filtered peanut oil, daily for the duration of the experiment. *In vivo* luciferase activity was assessed 4 hours after administration of TSA.

2.5. Whole body imaging

In vivo luciferase activity from tissues was analysed at set time points post-transfection as follows: 80 μ l of 30 mg/ml firefly luciferin (Biosynth, Basil, Switzerland) was injected i.p.

and intratumourally where appropriate. Mice were anaesthetised as before. Ten minutes post-luciferin injection, live anaesthetised mice were imaged for 3 min at high sensitivity using an intensified CCD camera (IVIS Imaging System, Xenogen, Caliper Life Sciences, England). Exposure conditions were maintained at identical levels so that all measurements would be comparable. All data analysis was carried out on Living Image 2.5 software (Xenogen). Luminescence levels were calculated using standardised regions of interest (ROI) for all three anatomical areas. Actual levels were obtained by subtracting the corresponding ROI of an untransfected mouse to account for background luminescence. For comparison between plasmids, luminescence was represented as p/sec/cm²/sr/plasmid copy.

2.6. Assessment of plasmid DNA in liver tissue using PCR analysis

To determine the presence of plasmid DNA in liver tissue, pCMV-Luc was delivered to the livers of 9 mice using electroporation as previously described. Luciferase expression was assessed by IVIS imaging at the time of sampling, 24 hr, 3 days and 10 days post treatment. Livers from three mice were excised at each time-point and snap frozen in liquid nitrogen. Livers were homogenized in TRIZOL Reagent (Invitrogen) using an Ultra Turrax T25 homogeniser (IKA Werke GmbH & Co. KG, Staufen, Germany) and total DNA was extracted as per the manufacturer's protocol. The presence of the plasmid DNA in the total DNA was determined by PCR using *luciferase* specific primers (For- 5'-AATCCATCTTGCTCCAA-CAC-3' Rev- 5'-ATCTCTTTTCCGTCATCGTC-3'). PCR conditions were: Initial denaturation at 95 °C for 15mins followed by 35 cycles (95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min) and a final extension of 10 m at 72 °C. The resulting PCR products were analyzed on a 1 % agarose gel.

2.7. Statistical analysis

The primary outcome variable of the statistical analyses was luminescence per cell per gene copy administered in each cell line or luminescence per gene copy administered in each organ measured at each time point. The principal explanatory variables were the delivery modalities used. *In vivo* luminescence was analysed as continuous. At specified time points, a two-sampled t-test was used to compare mean luminescence per gene copy administered for each delivery modality. Microsoft Excel 11.0 (Microsoft) and GraphPad Prism Version 4.0 (GraphPad Prism Software Inc, San Diego, CA, USA) were used to manage and analyze data. Statistical significance was defined at the standard 5 % level.

3. Results

Plasmid DNA encoding the luciferase gene transcribed from either the CMV (pCMV-luc) or Ubiquitin-B (pUb-luc) promoter was delivered to murine liver or quadriceps muscle by *in vivo* electroporation. Live whole body imaging (IVIS) was performed at various times over 370 days to determine luciferase expression. Expression mediated by the CMV promoter in liver, while initially high, reduced rapidly to background level by day 7 (figure 1a). When

the Ub promoter plasmid was examined in livers (figure 1b), luminescence was initially low but increased during the first week post transfection, before decreasing slowly, and remained higher than the CMV levels up to day 25. To examine other viral promoter activity in liver, pGL3 (SV40 promoter) was assessed (figure 1c). Like pCMV-luc, pGL3 displayed significantly faster reduction in expression than pUb-luc. A different temporal pattern of expression was observed in muscle for both the CMV and Ub promoters. Although promoter activity fluctuated over the period examined, a gross reduction in expression over time was not observed in this tissue with either CMV or Ub promoters (figure 1).

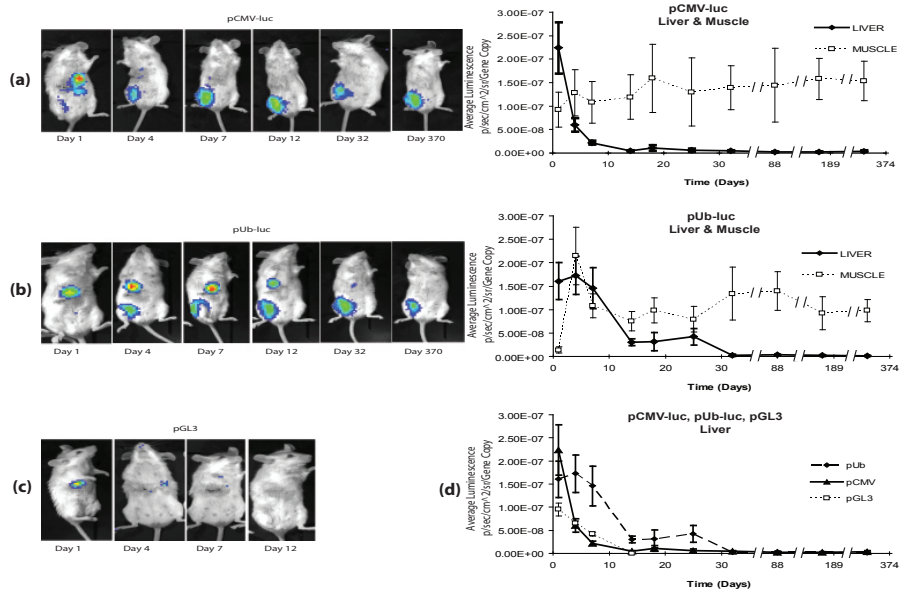


Figure 1. Duration of CMV and Ub promoter activity *in vivo* in liver and muscle pCMV-luc, pUb-luc or pGL3 was delivered to liver and quadriceps muscle (n=8) by electroporation and luminescence analysed *in vivo* over time using IVIS imaging. **(a)** CMV activity in liver was initially high, reducing to background levels by day 7. **(b)** Initial Ub activity levels were lower than those detected for the CMV promoter but increased and remained higher than that detected for CMV at later time points up to day 25. A gross reduction in expression over time was not apparent in muscle with either CMV or Ub promoters. **(c)** SV40 expression in liver decreased to background levels by Day 7. **(d)** Expression levels from both viral promoters (CMV and SV40) decline significantly faster than Ub in liver.

The kinetics of CMV, Ub and SV40 promoter activity were also analysed in tumour bearing mice. pCMV-luc, pUb-luc or pGL3 DNA was electroporated to subcutaneous (s.c.) JBS fibrosarcoma tumours upon reaching 80 mm³ in volume. IVIS imaging over 18 days (the lim-

it of tumour monitoring before animals required culling) demonstrated that the initially high expression driven by the CMV promoter was rapidly reduced to background level by day 4-post transfection (figure 2). Reduction was also observed with SV40 promoter, albeit with a heterologous temporal expression pattern to CMV, with pGL3 expression peaking at day 4 before rapidly reducing to background levels. Ub promoter activity was still evident at the final time point. pCMV-luc and pGL3 displayed statistically similar ($p = 0.98$) maximum to minimum rates of silencing (2.9×10^{-7} p/sec/cm²/sr/gene copy per day), higher than that of pUb-luc (6.8×10^{-8} p/sec/cm²/sr/gene copy per day). pCMV-luc expression was also found to rapidly reduce in s.c. human MCF7 breast carcinoma tumours growing in athymic mice (data not shown). Ubiquitin-B promoter transcriptional activity may be related to the normal functions of ubiquitin in cells, which is expressed constitutively for removing abnormal proteins and for modification of histones leading to gene activation, and so may not be subject to the down-regulation observed with many viral promoters (Ciechanover et al. 2000; Yew et al. 2001). Ubiquitin is also induced in response to cell stress, and expression might be up-regulated in response to cellular necrosis and apoptosis, which is especially relevant in growing tumours. Given that pUb-luc expression is evident long after viral promoter activity diminishes (up to day 25 for pUb-luc as opposed to day 7 for pCMV-luc and pGL3; figure 2), it is plausible that viral promoter plasmids remain present in liver post cessation of expression.

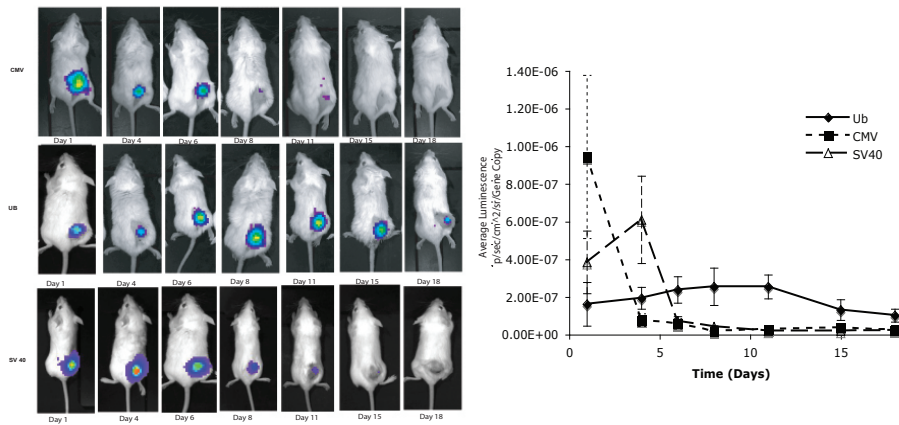


Figure 2. Duration of viral and native promoter activity in tumour pCMV-luc, pGL3 or pUb-luc was delivered *in vivo* to growing tumours ($n=6$) by electroporation and luminescence analysed *in vivo* over time using IVIS imaging. Expression from both viral promoters (CMV and SV40) rapidly diminished, whereas Ub promoter activity was still evident at the final time point (day 18) when mice required culling due to tumour size. Ub mediated expression levels were at 39.4 % of maximal level on final time-point, compared with 2.4 % and 3.5 % for CMV and SV40 respectively.

To test for the presence of plasmid, DNA was extracted from murine livers at various times post transfection with pCMV-luc and PCR analysis performed. DNA PCR results from days 1, 3 and 10 confirmed the presence of *luciferase* DNA in tissue after cessation of expression, suggesting that inhibition of transgene expression occurred at the level of or post transcription (figure 3). Our findings indicate that both viral promoters examined provided short-lived expression in tumours and liver, whereas use of Ub promoter significantly prolonged transgene expression. Importantly, we also found that viral promoter activity was dependent on target tissue, since no reduction in expression was observed in plasmid electroporated muscle with both viral and mammalian promoters.

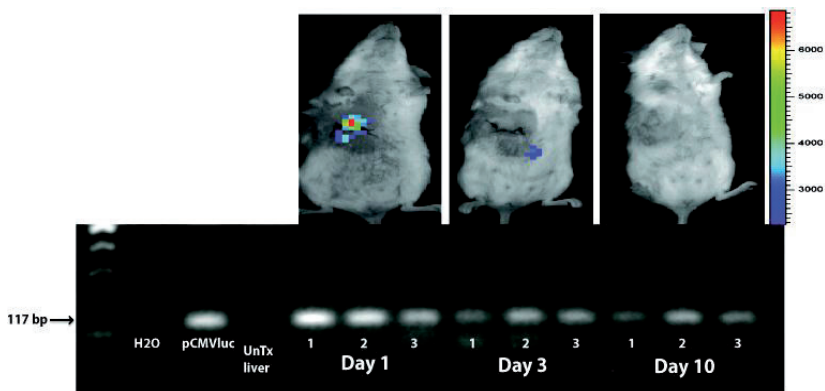


Figure 3. Plasmid DNA persists in liver after cessation of expression PCR analysis of DNA extracted from murine livers (n=3) on days 1, 3 or 10-post electroporation with pCMV-luc. A representative mouse from which DNA was extracted at each time-point is shown. PCR using primers specific for the *luciferase* gene indicates presence of plasmid. Untransfected liver samples did not yield PCR product.

In order to examine any effects of T-cell mediated immune activity on viral promoter construct expression, pCMV-luc expression in livers of athymic mice was examined. No difference in the magnitude or duration of expression was observed between immune competent Balb/C and T-cell deficient mice, suggesting that cellular immune responses were not involved in the observed reduction in hepatic expression of pCMV-luc (figure 4a). Other studies have indicated that luciferase protein has low immunogenicity, and immune-mediated destruction of luciferase-producing cells does not occur in mice (Davis et al. 1997), while the persistence of expression in muscle here also makes this unlikely as a cause for silencing in other tissues. The observation of indefinite expression in plasmid electroporated muscle is in direct contrast to Ad expression in quadriceps muscle, which has been shown to be eliminated through T cell and antibody immune activities and/or CMV promoter methylation (Jooss, Yang et al. 1998; Brooks et al. 2004).

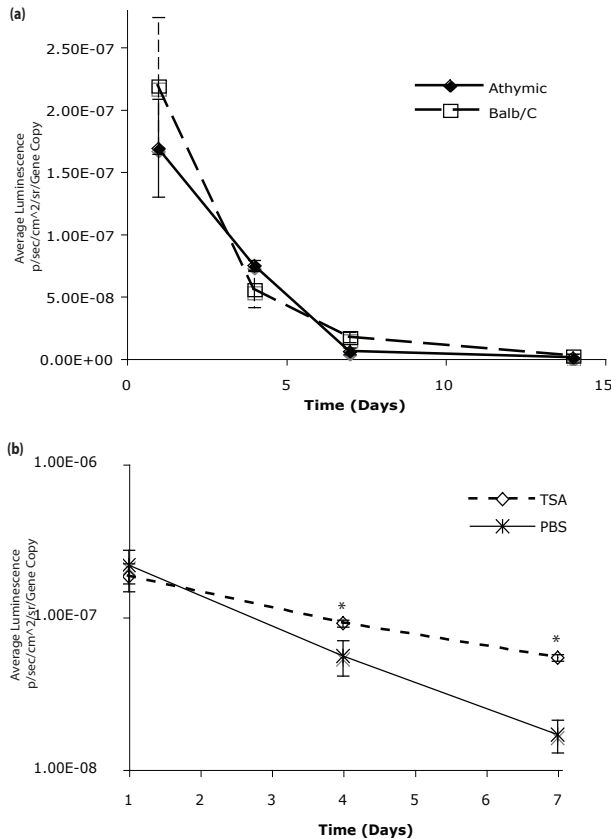


Figure 4. (a) pCMV-luc is silenced in livers in absence of T cells pCMV-luc was electroporated *in vivo* to livers of athymic mice and IVIS imaged (n=4). No difference was observed in plasmid expression at any time point when compared with expression in immunocompetent Balb/C mice ($p > 0.25$). **(b) Effect of deacetylation agent on pCMV-luc expression *in vivo*** pCMV-luc was delivered to livers by electroporation (n=4). TSA or PBS was i.p. administered daily. Gene expression was analysed using the IVIS imaging system. The magnitude and duration of gene expression in animals treated with TSA was significantly increased. * denotes statistically significant difference between groups, $p < 0.05$.

4. Discussion

We did not determine the reasons for the observed tissue-specific nature of viral promoter silencing, and it remains unclear as to why liver and tumour, but not muscle, affected plasmid expression. Plasmids function predominantly in an episomal fashion and copy number per cell is reduced proportional to cell replication. As such, genes would be expected to be

diluted rapidly in tissues with a high mitotic index. Liver hepatocytes and skeletal myocytes are fully differentiated and have a low turnover, unlike tumour cells. (Ayers & Jeffery 1988) It may be hypothesised that the static nature of cell turnover in muscle compared with tumour is relevant in this context. However, this cannot fully account for the observed loss of expression, since in our study, the rate of reduction of expression for plasmids with promoters of mammalian and viral origin was different. Also, previous studies have shown no alteration in longevity of transgene expression when cell turnover was inhibited (Herweijer et al. 2001). Furthermore, we demonstrated by PCR that pCMV-luc persisted in liver cells after expression ceased. We think it is unlikely that the reduction in pCMV-luc expression was due to a parallel reduction in the plasmid DNA as this was not seen for the Ub promoter where similar plasmid copy numbers would be expected.

It has previously been demonstrated that plasmid transgene expression can be modulated with chromatin remodelling agents (Bartoli, Fettucciari et al. 2003). To this end, murine livers were electroporated with pCMV-luc and mice systemically administered the histone deacetylase inhibitor trichostatin-A (TSA) daily for the duration of experiment. TSA is a specific inhibitor for histone deacetylase (HDAC) and is known to enhance gene expression in viral and plasmid-transfected cells *in vitro* and *in vivo* (Vanniasinkam et al. 2006). It has been shown that HDAC binds to the CMV promoter, and TSA may act to overcome such transcriptional repression (Tang & Maul 2003). In our experiments, TSA administration significantly increased levels of expression at later time points, compared with control ($p < 0.002$ on day 7; figure 4b). Interestingly, a further increase was noted when 5' azacytidine (aza-C), a non-specific methylation inhibitor, and trichostatin were used in combination, while aza-C in isolation had no effect (data not shown).

While this study did not generate data to correlate RNA levels with luminescence, differences in transcription appears to be the key element in observed expression levels. Firefly luciferase protein is known to have a short half-life *in vivo*, in the region of 1 - 4 hours (Baggett et al. 2004; Tangney & Francis 2012), and any luminescence detected in our experiments was due to recently transcribed gene. Furthermore, given that pUb-luc expression is evident long after viral promoter activity diminishes (up to day 25 for pUb-luc as opposed to day 7 for pCMV-luc and pGL3; figure 1), it is likely that viral promoter plasmids remain present in liver post cessation of expression, and we demonstrated by PCR that pCMV-luc DNA was present in liver 10 days post transfection. There exist numerous reports linking viral promoter DNA methylation with transcriptional silencing in gene therapy settings *in vitro* and *in vivo* (Di Ianni et al. 1999; Brooks, Harkins et al. 2004; Al-Dosari et al. 2006).

Our findings are consistent with previous studies in lung tissue where the levels and duration of transgene expression *in vivo* were compared using plasmid vectors coding for the CMV or Ubiquitin promoters (Gill et al. 2001; Yew, Przybylska et al. 2001; Gazdhar, Bilici et al. 2006). Further specific methylation assays may elucidate the precise mechanism of viral promoter silencing here. Given that many tumour types have been shown to have abnormal methylation, this phenomenon may represent a serious hindrance to cancer gene therapy which use of native promoters may abrogate as demonstrated here (Kanai 2008). Furthermore, the finding of indefinite high-level expression in plasmid electroporated muscle irre-

spective of the promoter type has important therapeutic implications. Skeletal muscle is a large and accessible tissue, within which a plasmid-based gene therapy might be a safe and efficient method for systemic protein production, particularly when combined with either endogenous or exogenous regulatable systems. We have previously demonstrated the application of an inducible plasmid based system in *in vivo* murine tissue (Morrissey et al. 2012). In addition to providing an “off switch” to safeguard against side effects, this also allows optimal temporal delivery of therapeutic, tailored to when it can most efficiently achieve a biological response.

5. Conclusion

In summary these results highlight the importance of promoter, tissue and vector variables in achieving appropriate transgene expression for DNA therapeutic strategies.

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Silencing of Transgene Expression: A Gene Therapy Perspective

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Additional information is available at the end of the chapter

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1. Introduction

The treatment of a number of diseases can be achieved through gene addition therapy, where curative transgenes are established within the patient's cells after delivery with viral or non-viral vectors. The defective cells requiring treatment are typically differentiated; these cells or their progenitors can be targeted for therapeutic gene transfer. However, as the abundance of progenitor cells varies between different tissues and in the same tissue during the fetal, neonatal and adult stages of development, the scarcity of a particular progenitor cell pool, the paucity of spontaneous departures of progenitor cells down differentiation pathways and unclear differentiation induction conditions can complicate genetic therapeutic intervention via these cells. Nevertheless, gene transfer to progenitor cells can be a preferred option when differentiated cells are either poorly accessible for the vector or, once differentiated, are defective beyond repair by gene therapy. Genetic conditions with considerable value in therapeutic gene transfer to progenitor cells include cystic fibrosis (CF) and severe combined immunodeficiency (SCID).

The delivered transgenes can integrate into the chromosomal DNA, replicate episomally or persist as non-replicating episomal elements in non-dividing cells. Depending on the properties of the transgene expression cassette, particular features of specific transgene integration sites and the state of the individual recipient cells, the transgenes are expressed with varying degree of efficiency. On some occasions, the transgenes are permanently silenced immediately after introduction, on other occasions transgene silencing occurs only after a certain period of adequate expression and on still other occasions transgene expression varies dramatically among the individual clones of transgene-harbouring cells. Such variation is thought to be mainly due to the transgene's interaction with its immediate genetic neigh-

bourhood within the host genome; a phenomenon, which is similar to 'position effect variegation' in normal development caused by spontaneous clone-wise silencing of some resident genes [1]. Typical position effect variegation is epigenetic instability and should be distinguished from variegation due to somatic mutations, e.g. due to variations in the length of polynucleotide repeat expansions [2] or due to the sorting of mitochondrial genomes in mitochondrial heteroplasma [3]. The element of randomness, which is inherently present in position effect variegation, should not come as a surprise. In fact, stochastic fluctuations of gene expression are typical both at the level of variation between different cells of tissue and at the level of temporal variation within one cell. Both of these modes of variation are essential for normal differentiation and tissue-patterning with the input of stochastic variation being decisive when a developmental signal is present at a near-critical level. For the gene therapist, it is important that the permanent silencing of transgene expression can occur both in postmitotic target cells and target cells undergoing clonal expansion, while variegation is typically associated with clones of dividing cells. Stable long-term transgene expression in differentiating cells is particularly challenging. In fact, the introduced genes are subject to the pre-existing and developing gene expression patterns in the target cells, which can override the signals from the transgenes' own regulatory elements and, thus, can cause transgene expression shutdown. Indeed, at a transcriptional level, the changing scenery of transcription initiation factor pools, chromatin re-modelling and DNA methylation events during differentiation contribute to the transiency of transgene expression.

Genomes in general and, in particular, mammalian genomes have a mosaic organisation with functionally related genetic elements often being in close physical proximity. There are three teleological reasons for this: 1) expediency of genetic exchange; 2) straightforward temporal control of gene expression; 3) economy of energy, enzymes and other factors serving the genetic elements. The second and the third of these reasons are also sufficient for the existence of a finely patterned 3D-arrangement of DNA in interphase nuclei, simplifying the functional interactions between distant genetic elements, e.g. interactions regulating gene expression. It is intriguing to propose that the need to orchestrate gene expression in time and the economy need are also driving the astonishing interconnectedness of all gene silencing mechanisms, which we shall address in this chapter.

The gene therapist should take advantage of the pre-existing regulatory moduli present in the target cells and should also supply the transgenes with their own expression control elements. The regulatory elements required for reliable, long-term and tissue-specific transgene expression include minimal promoters, enhancers, regulatory introns and locus control regions. The functional arrangement of all these elements is ultimately achieved in 3D. This should be borne in mind, when 2D assemblies of regulatory elements are called 'promoters'. Some 'promoters' are, in fact, motley artificial chimeras. For example, a fusion between a human cytomegalovirus (CMV) immediate-early enhancer and chicken beta-actin promoter, exon1 and intron1 is called 'CBA promoter' or 'CAG promoter' [4].

In general, in the majority of situations in gene therapy, transgene silencing and variegation are undesirable. We review here different factors, both host-dependent and vector-dependent, which are known to contribute to silencing and variegation of transgene expression and

which should be taken into account where choosing or designing effective gene therapy vectors and strategies for their administration.

2. Host genetic factors of silencing and position effect variegation

Patterns for maintaining gene repression or activation are governed by regulatory machinery acting at multiple levels: 1) transcription; 2) mRNA processing, export from the nucleus, translation and degradation; 3) protein folding, modification, transport and degradation. Control of gene expression is well-coordinated and highly hierarchical, with the control of transcription initiation situated at the top of the regulatory ladder. A number of interacting instruments of transcriptional gene activation and silencing in mammals are known: DNA methylation (e.g. methylation within CpG-islands of promoters), amino acid sequence variants of histones, covalent modifications of histones, histone-binding proteins (e.g. powerful inhibitors of gene activity from the Polycomb Group) and combinations of transcription initiation factors specific for particular tissues and developmental stages. The pivotal point is the access of the transcription machinery to DNA, which is regulated via DNA methylation and chromatin remodelling. With some simplification, it can be generalized that 'coarse tuning' of gene expression (e.g. long-term silencing) is provided by DNA methylation, 'medium tuning' is provided by chromatin remodelling and 'fine tuning' is achieved via various transcription factors and a multitude of other regulatory devices.

The various branches of the regulatory machinery play their own particular roles and yet are inherently interconnected. As detailed below, a prime example of this is the deep involvement of the miRNA pathway both in mRNA degradation and in the establishment of chromatin methylation patterns [5].

2.1. The role of DNA methylation in silencing

DNA methylation is an important epigenetic mark involved in cell differentiation and organ and tissue development, which plays a crucial role in the establishment of genomic imprinting (parent-dependent silencing of alternative alleles) in both male and female germ lines. However, in gene transfer experiments, the methylation of transgenes was shown to be just one ingredient in the dynamic interplay of various factors responsible for silencing and variegation [6].

De-novo methylation patterns in humans are established mainly on implantation and in gametogenesis. Two DNA (cytosine-5)-methyltransferases, DNMT3A and DNMT3B, play an essential role in *de-novo* methylation while DNMT3A in cooperation with the auxiliary protein DNMT3L is responsible for imprinting. There is still much we do not know about the manner in which the inactive state of the imprinted chromosomal domains is achieved and what factors trigger this type of silencing. The available evidence indicates that 'Smc hinge proteins' can be particularly important in epigenetic silencing [7]. Thus, in studies based on X-linked GFP transgene silencing, the SmcHD1 gene was shown to play a critical role in X-chromosome inactivation in mammals [8,9]. The recruitment of SmcHD1 to the X-chromo-

some may involve the non-coding Xist RNA, proteins from the Polycomb group and DNA methyltransferases [7].

An area of intriguing research is the relationship between DNA hypermethylation and the function of locus control regions (LCRs), controlling the local state of chromatin [10,11].

2.2. The role of histone variants and histone modifications in silencing

There are two types of structural variations among histone molecules. Firstly, there are low abundance species of histones with unusual amino acid sequences, so-called histone variants. Secondly, histones are amenable to standard covalent protein modifications such as acetylations and methylations of specific amino acid residues. Both structural variations are known to play important roles in the regulation of gene expression activity.

Regions of constitutive heterochromatin are particularly prone to encroaching on the transgene in a variable pattern in different cells and, thus, to interfering with transgene expression. Different loci in human chromosomes have a variable tendency to become involved in heterochromatin structures. For example, chromosomes' centromeres and telomeres are typical regions of heterochromatin, which are known to expand occasionally, inducing steady or intermittent silencing. In the case of centromeres, the silencing machinery might involve the histone variant CENP-A, which is found exclusively in centromeres. Other histone variants could also play a role in silencing. Thus, the histone variant macroH2A appears to be important in gene silencing on the inactive X-chromosome. In contrast, the histone variants H2A.Z and H3.3 are known to be conducive for transcription.

DNA methylation and histone modifications are closely linked to chromatin remodelling and are often jointly implicated in gene silencing and position effect variegation. Using an *in vivo* mammalian model for position effect variegation, Hiragami-Hamada and co-workers [12] extensively investigated the molecular basis for the stability of heterochromatin-mediated silencing in mammals. Comparison between two transgenic lines, containing different numbers of copies of human CD2 transgenes integrated within or close to a block of the pericentric heterochromatin, revealed that the variegation of CD2 expression is indeed associated with both genomic DNA methylation and histone modifications such as H3K9me3. However, DNA methylation was the key modification that accompanied the formation of an inaccessible chromatin structure and more stable gene silencing [12,13].

2.3. Silencing mediated by Polycomb proteins

Silencing can be mediated by proteins from the Polycomb group (PcG). These proteins can form giant complexes, which are tethered to histones and regulatory DNA sequences called Polycomb Response Elements (PREs). When the PcG proteins bind histones, they suppress all the gene expression activity in the respective area of chromatin. In mammals, PcG proteins are known to be involved in cell differentiation and tissue formation and also to contribute to tumorigenesis, genomic imprinting, stem cell maintenance and aging [14-16]. The emerging picture from fundamental research suggests that counteracting PcG repression can only be achieved by a combination of multiple inputs converging at chromatin [17]. Be-

sides the normal requirement for the recruitment of transcription factors and co-activators, the genomic targets of PcG proteins require the activity of specific demethylases and methyltransferases for the gene expression to proceed [18].

Importantly for gene therapy, PcG protein complexes have been recently demonstrated to be able to repress transcription activity in genomic repeats and some transgenes [19].

2.4. Tissue specific and developmental stage specific transcription factors

There are two types of transcription factors: 1) auxiliary proteins, which bind other proteins in the transcription complex; 2) DNA-binding sequence-specific transcription factors. The latter type can straightforwardly be recognised *in silico* by the observation of some distinct patterns within the DNA-binding domains of transcription factors, e.g. the zinc-finger motif, the helix-loop-helix motif or the leucine-zipper motif. *In silico* analysis, e.g. using *Biobase* software (<http://www.biobase-international.com>), is currently also a method of choice for pinpointing transcription factor binding sites and, therefore, for predicting gene expression activation patterns.

2.5. Silencing mediated by non-coding RNAs

It has become clear that non-coding RNAs have an important bearing on gene and transgene expression. In general, there are several mechanisms for the regulatory effects of non-coding RNAs in gene expression. The two most important control points appear to be the direct regulation of transcription initiation and the regulation of mRNA degradation through RNAi by miRNAs. Recent findings revealed that non-coding RNAs are critical factors in the recruitment of PcG members to the cell chromatin [20,21]. At the same time, the miRNA pathway turned out to be significant in establishing the DNA methylation and histone modification patterns [5,22].

In animals, small RNAs, namely piRNA species, which are typically 24-32 nucleotides in length, have been shown to mediate genomic DNA methylation. These non-coding RNAs associate with Piwi clade proteins from the Argonaut superfamily and act analogously to the well-documented RdMD complexes in plants. The primary role of piRNA in many animals appears to be the silencing of retrotransposons via DNA methylation in germ lines. In fact, the lack of transposons' suppression in spermatogenesis often results in defects and the loss of germ cells with age. Although it is not clear whether the same mechanism is responsible for the protective silencing of viral genomes after viral infections of mammalian cells, the small RNAs are likely to be involved in *de-novo* methylation of viral DNA through a similar mechanism. Thus, small noncoding RNAs could potentially provide a flexible regulatory link between transgene recognition, PcG proteins recruitment and transgene silencing through DNA methylation, histone modifications and chromatin remodelling.

It appears that, in general, regulation via RNAi has a smaller long-term influence on gene expression than histone modifications and DNA methylation, acting rather as a rapid response system. Indeed, it would be too energetically inconvenient for cells to synthesize mRNA and then to destroy it on a permanent basis.

3. Gene vector properties, which are known to contribute to transgene silencing

Long-term transgene expression is highly desirable for most gene therapy applications. However, it is a relatively common occurrence for transgene expression to die out both in terms of the decrease of the efficiency of expression in individual cells and in terms of the reduction of the fraction of expressing cells.

A wide variety of vectors can be used for the delivery and establishment of transgenes and their control elements. Some of the vectors, so called 'viral vectors', are generated using a top-down approach by piggy-backing on the natural gene transfer machinery of viruses. In contrast, 'non-viral' vectors are either pure nucleic acids or synthetic nano-particles, which are generated using a bottom-up strategy. A pivotal feature of any gene therapy vector (with the obvious exception of cytoplasmic-only vectors such as mRNA-based vectors) is the final localization of the delivered transgenes in the nuclei of the target cells. In general, transgenes can be integrated into random chromosomal sites, integrated into pre-selected chromosomal sites and/or left to exist episomally. Specialized molecular machinery for efficient random integration is born by retroviral vectors [23], lentiviral vectors and eukaryotic transposon vectors. Although the bulk of the DNA delivered with non-transposable plasmid, minicircle and PCR-generated vectors stays episomally, some of the vector DNA also randomly integrates into the chromosomal DNA. The genetic neighbourhood at a transgene integration site has an important bearing on the temporal profile of transgene expression. Nevertheless, many factors that determine the susceptibility of transgene to silencing are defined by the properties of the employed vector, transgene and co-introduced expression control elements.

Multimeric transgene inserts were reported to induce silencing [24]. Unfavourably, even if a gene vector delivers monomeric DNA, spontaneous chromosomal integrations often result in vector DNA multimers (it remains unclear whether the multimers are formed before or after the initial integration event). Silencing due to repetitive DNA was also demonstrated when the introduced DNA contained trinucleotide repeat expansions [25]. This result has an implication for the gene therapy of recessive polyglutamine diseases, as therapeutic transgenes can contain triplet expansions of some minimal length. The precise mechanism for silencing through the recognition of multimeric transgenes and trinucleotide repeats in the host genomic DNA still remains unclear.

Transgene silencing is often blamed on the malfunction of foreign gene expression control elements. Indeed, this phenomenon is sometimes referred to as 'promoter shut down'. Certainly, different promoters vary in their ability to maintain long-term transgene expression in specific cell populations. In particular, there is a clear tendency for some promoters to turn off in cells where they are not normally active. The mechanisms for such effects can be quite indirect. Thus, the ubiquitous CMV promoter can activate transgene expression in antigen-presenting cells with the ensuing immune response and elimination of all vulnerable transgene expressing cells [26].

Some bacterial plasmid backbones are known to cause transgene silencing [27-29]. In addition, bacterial plasmid backbones interfere with gene delivery into human cells after DNA administration *in vivo* because of the innate TLR9-receptor-mediated immune reaction to unmethylated bacterial 'CpG-motifs' within these backbones. In an attempt to alleviate the immune reaction, methylation of these sequences *in vitro* was attempted. Disappointingly, on some occasions the methylation of plasmid gene vector DNA resulted in increased silencing of transgene expression [30]. The depletion or ablation of CpG motifs from bacterial plasmid backbones is known to substantially reduce their immunogenicity. The effects of CpG-depletion and ablation on transgene silencing are expected, but the available data on this issue are currently quite limited.

Bacterial lipopolysaccharides (LPS) often co-purify and contaminate plasmid gene vector DNA. These endotoxins can substantially reduce the efficiency of transfection *in vitro* [31,32] and *in vivo*, where LPS are known to induce a TLR4-receptor-mediated innate immune response. Bacterial endotoxins exhibit a profound effect on cellular regulatory networks [33]. Therefore, it is possible that tilting cells towards 'transgene-silencing mode' is an important contributing factor in the endotoxin-mediated inhibition of transfection.

4. Therapeutic gene vectors and the strategies for their use, which are employed to avoid transgene silencing

Stable long-term transgene expression depends on the intertwined issues of reliable maintenance of transgenes in target cells and a robust policy to prevent undesired transgene silencing. In general, these two issues are to a large extent under the control of the gene therapist, as both of them can be addressed through the gene vector design and the delivery mode. The regulation of gene expression in eukaryotic cells is exceptionally complex and multi-faceted. As a result, the strategies used to achieve sustainable transgene expression should address multiple possible reasons for the transgene expression shutdown.

4.1. Employment of cytoplasmic-only (non-nuclear) vectors

As most silencing mechanisms are nuclear-based, gene vectors with direct cytoplasmic expression, which are not required to enter the nucleoplasm, are well-positioned to avoid silencing. Thus, non-viral mRNA vectors [34] or positive strand RNA-based viral vectors such as Sendai virus based vectors [35] can be employed. In addition to the escape from silencing, the advantages of extra-nuclear-delivery vectors include relatively fast transgene expression and the absence of potentially mutagenic genomic insertions. The downside is that transgene expression using such vectors is never long-term because of the eventual degradation of RNA in cells and because of RNA dilution in the dividing cells. Moreover, the fundamentally low fidelity of RNA replication undermines efforts to generate artificial vector systems with replicating RNA episomes. The key upside is that low immunogenicity and minimal toxicity of such vectors accommodate their repeated administration well.

4.2. CpG ablation, CpG depletion and minimized DNA vectors

The methylation of chromosomal DNA is one of the most powerful mechanisms for the shut-down of gene expression. Thus, the design of gene therapy vectors should take into account the amenity of the vector sequences to methylation. Firstly, the purposeful exclusion of entire methylation-prone CpG islands should be considered. Secondly, CpG-depleted or CpG-ablated modules, produced through the point-wise replacement or removal of CpG dinucleotides, should be taken advantage of. The generation of functionally active CpG-ablated sequences is fairly laborious; the CpG-ablated gamma replicon from the bacterial plasmid R6K and some antibiotic-resistance genes are available from *Invivogen*.

Clearly, as repetitive sequences are known to induce silencing, their use in therapeutic gene vectors should be avoided as far as possible.

A common way to reduce the chances of transgene silencing is to shorten the auxiliary vector sequences outside of the therapeutic transgene expression cassette. For example, the plasmid selection markers can be very short indeed [36]. In fact, a plasmid replication origin can be re-utilised as a plasmid marker using the 'plasmid addiction' phenomenon [37].

The trend to exclude unwanted sequences from gene transfer vectors led to the generation of specialized minimized DNA vectors. The most tested versions of such vectors are DNA fragments amplified *in vitro* using polymerase chain reaction (PCR) [38], plasmid-derived linear terminally looped 'midges' [39] or circular supercoiled 'minicircles' [40]. Minicircle vectors are produced by intramolecular site-specific recombination within bacterial plasmids. The superior efficiency of gene delivery and the longevity of transgene expression achieved with minicircle DNA was observed in multiple studies (e.g. [41]). The production of minimized DNA vectors is a biotechnological challenge. For example, advanced methods and bacterial strains were developed for efficient bacteria-based minicircle DNA production. The generation of PCR amplicons with Taq-polymerase is relatively inexpensive. However, the load of Taq-polymerase-introduced mutations may make one consider alternative *in vitro* amplification methods for the large-scale synthesis of double-stranded DNA, e.g. ligase chain reaction (LCR), which is based on the ligation of preassembled oligonucleotides.

The usual aim in the production of minimized DNA vectors is the removal of sequences of bacterial origin, such as plasmid backbone sequences, as they can be immunogenic and some of them were reported to cause silencing [27,29]. It should be emphasized that transgene silencing through the co-delivery of specific plasmid sequences should not be generalized to all plasmid sequences and each plasmid sequence or bacterial sequence needs to be tested individually. More research is required to identify the affected bacterial replicons and to pinpoint the mechanism for the induction of silencing by bacterial DNA sequences. Another avenue is the development of novel specialized forms of minimized vectors, such as 'minivectors' for RNAi-based therapy [42].

4.3. Judicious choice of tissue-specific, inducible and ubiquitous promoters to control transgene expression

Promoters are the gene expression control elements, which are typically co-introduced with therapeutic transgenes. In scientific literature, the word 'promoter' is often an umbrella term, which in addition to a minimal promoter also incorporates other linked genetic elements such as enhancers, transcription factor binding sites and even regulatory introns. Promoter is a key element of the regulatory machinery required for long-term non-silenced transgene expression. Different promoters vary in their strength, tissue specificity, specificity for particular developmental stages and ability to react to external stimuli (inducibility). Each therapeutic setting requires a thoughtful choice of a transgene promoter. Thus, some ubiquitous promoters are appropriate for consistent long-term transgene expression in differentiating stem cells passing through a number of developmental phases [43]. Ubiquitous promoters are also appropriate in situations where the resident homologue of the therapeutic gene is naturally expressed ubiquitously [44]. Tissue-specific promoters have been known for a long time to be instrumental for long-term transgene expression in terminally differentiated cells in the liver, vascular tissue, muscle and central nervous system [45]. Inducible promoters are appropriate where the constitutive expression of the therapeutic transgene is undesired and/or where bespoke activation of the therapeutic transgene is required. In addition to the heavily used tetracycline-sensing promoter systems, inducible promoters can be activated by heat, light and gas-born acetaldehyde [46]. Clearly, the construction and determined exploitation of new hybrid promoters can resolve many issues in transgene silencing.

4.4. Multiple transgene insertions into random chromosomal sites

Random integration of transgenes into chromosomes is typical for a number of gene delivery systems. Spontaneous chromosomal integration of vector DNA within target cells is not efficient. Thus, enhanced random chromosomal integration of plasmid gene vectors can be attained using genetic elements of eukaryotic transposons, retroviruses or lentiviruses (lentiviruses form a subgroup of retroviruses with a somewhat larger genome and the ability to infect non-dividing cells). However, many integration events occur in unfavourable genetic neighbourhoods resulting in the silencing of the respective copies of the transgenes. Hence, position-dependent silencing means that individual transfected or transduced cell clones differ in terms of the longevity of the transgene expression. Random chromosomal integration of transgenes tend to occur in transcriptionally active areas of the genome where heterochromatin condensation and DNA methylation are unlikely to interfere with transgene expression. However, as cells differentiate, the pattern of heterochromatinization and DNA methylation changes and some of the transgenes find themselves in transcriptionally silent areas of the genome. Therefore, the shutdown of transgene expression is particularly common in cell populations undergoing differentiation. In these circumstances, it is certainly possible to increase the chances of long-term transgene expression by increasing the number of randomly chromosomally integrated transgenes through a higher concentration of vector and/or repeated rounds of vector administration. Thus, the gene therapist can aim to gener-

ate multiple copies of transgenes, indiscriminately integrated within the target genome, hoping that at least one of the copies will reside in a suitable chromosomal site that will be immune to silencing.

The employment of transposable genetic elements for efficient random integration of therapeutic transgenes was complicated by the fact that mammals do not have their own active or easily re-activatable transposons. Therefore, a number of heterologous transposons were adapted for use in human cells. Recombination machinery from Sleeping Beauty, PiggyBac, Tol2 and Mos1 transposons was shown to be capable of directing chromosomal integration of transgenes [47]. Genes for transposases were either included within the cargo gene vector plasmid or were delivered into human cells on a separate plasmid. Mutant transposases with enhanced activity for random DNA integration were developed.

A caveat of the anti-silencing strategy relying on multiple transgene insertions into random chromosome sites is a possibility of potentially deleterious or tumourigenic mutations due to insertional mutagenesis. However, this drawback is irrelevant for highly differentiated and non-dividing cells where, firstly, only a limited set of gene products is required for cell survival and functional competence and, secondly, only a minimal risk is present for the selection of malignancies. In fact, many terminally differentiated cells are either polyploid or polynucleated; both of these statuses can alleviate the impact of insertional mutagenesis.

4.5. Site-specific chromosomal integration

One of the ideal scenarios, where transgene silencing is avoided, involves the transgene DNA being site-specifically integrated into a 'benign', silencing-resistant chromosomal site where there is little chance of transgene consumption by heterochromatin. Thus, targeting transgenes to a continuously active chromosomal locus can resolve the transgene expression shutdown problem. In particular, sites could exist within chromosomal DNA, where an integrated transgene would be immune to chromatin re-arrangements and other regulatory events during differentiation. A possible candidate site is the human homologue of mouse Rosa 26 locus, which is being successfully used to express various transgenes in mouse transgenic studies.

In principle, both transposases and retroviral integrases can be re-engineered into site-directed recombination enzymes through their fusion with appropriate site-specific 'tethering' domains [48]. In addition to tethered transposases and retroviral integrases, the site-specific integration of transgenes into human chromosomes can be achieved via the modification of *bona fide* site-specific recombination systems.

Site-specific DNA recombination systems are comprised of recombinase enzymes, their co-factors and their cognate recombination sites. Site-specific recombination systems can be classified into two general types: irreversible and reversible ones.

Site-specific recombination machinery for irreversible recombination is typically borrowed from the chromosome integration systems of temperate bacteriophages. In integrative recombination systems there are two types of recombination sites, which are normally referred to as *attP* and *attB*. An archetypical example is bacteriophage lambda integrase (Int)

catalysing a one-off recombination event between the lambda's *attP* site and the chromosomal *attB* site. The reverse reaction, excision of prophage, is often possible; however, a separate enzyme or a separate subunit of bacteriophage integrase is normally required to catalyze the excision. The *attB* sites are typically shorter than the corresponding *attP* sites. Thus, in the recombination system from the *Streptomyces coelicolor* bacteriophage phiC31, *attP* is 39 bp long and *attB* is 34 bp long. Similarly, the recombination system from the *Lactococcus lactis* bacteriophage TP901-1 has 50 bp long *attP* and 31 bp long *attB*. Consequently, in artificial recombination systems within the mammalian setting, higher specificity of integration is achieved with longer *attP* sites positioned within the chromosomal loci. It has turned out that the human genome contains a close analogue of the phiC31 *attP* site. Extensive mutagenesis of the phiC31 integrase gene has produced versions of the enzymes with very high specific activity towards this native human site [49]. Cell-permeable and nuclear targeted versions of phiC31 integrase were also created, these recombinant enzymes can be used to create transient, 'hit-and-run', recombinase activity in human cells that is required for the stable integration of therapeutic transgenes.

The typical original *in vivo* function of the reversible site-specific recombination systems is to preserve the monomeric status of a plasmid, prophage or episome via the resolution of circular DNA multimers to monomers; monomeric status is important for the maintenance stability of many plasmid replicons. Commonly used reversible systems include bacteriophage's P1Cre recombinase with its cognate *loxP* sites and FLP recombinase (flipase) with its cognate *FTR* sites from the yeast *Saccharomyces cerevisiae* '2-micron circle' episome. Many reversible systems were successfully used for the chromosomal integration of transgenes in pre-engineered cells. However, it should be noted that some site-specific recombination systems are fundamentally unsuitable for chromosomal integration strategies. Thus, ParA resolvase and *MRS* sites from the plasmid RK2 constitute a reversible system for intramolecular recombination; however, in this system there is no molecular recombination between *MRS* sites situated on separate DNA molecules.

Of course, the employed bacterial recombination systems have to be functional in eukaryotic cells [50]. A potential pitfall to be aware of is that some of the site-specific recombinases require an additional co-factor; e.g., IHF (integration host factor) is an obligatory element for lambda Int/*attB*/*attP* system. Unexpectedly and encouragingly, at least on some occasions mammalian cells are able to provide suitable co-factors [50].

The wild type human adeno-associated virus type 2 (AAV2) is the only known human virus capable of site-specific chromosomal integration. AAV2 uses the chromosome-tethering strategy for genomic insertions. Expression of the Rep gene is required for integration of the viral genome into a unique DNA sequence within specific chromosomal loci. The Rep proteins of this virus bind both several Rep Binding Sites (RBS) within the viral DNA and the RBS sites in the human genome (known as AAVS1, AAVS2 and AAVS3) leading to preferential integration of the viral DNA in the genomic loci 19q13.42, 5p13.3 and 3p24.3.

An important step forward in the exploitation of the site-specific integration system of AAV was achieved when the AAV Rep protein was used to direct the integration of integrase-defective retroviral vectors into human 19q13.42 locus [51]. The transfer of the locus-specific

chromosomal integration apparatus of AAV2 to other vector types, e.g., plasmid gene vectors, can be accomplished as well [52].

4.6. Episomal localisation of a transgene

Episomal maintenance of transgene expression cassettes is an attractive strategy to escape the control of some resident gene regulation systems, such as chromatin remodelling machinery, over transgene expression. The problem with this approach is that viral replicons, e.g., compact episomal replicons from SV40, polyoma, papilloma viruses, which are often completely adequate for the research use of gene vectors, are rarely acceptable for therapeutic applications. Indeed, the expression of the large SV40 T-antigen and, hence, the malignant transformation of the recipient host cells is required for SV40-origin-based replication. Similarly, EBNA1-oriP DNA segment of Epstein-Barr Virus (EBV) can be used to support the maintenance of plasmid gene vectors in the nucleoplasm of dividing laboratory cells. Although EBNA1 expression does not result in a typical malignant transformation, it can still tilt the cells towards undesired immortalisation [53].

Alternative benign episomal replicons are being sought. Encouragingly, the scaffold/matrix attachment region (S/MAR) from the human β -interferon gene was reported to support non-viral episomal replication when coupled to a promoter [54]. Thus, episomal maintenance mediated by S/MAR elements might be the reason behind the well-established beneficial effects of these elements on transgene expression [41,55,56]. Non-viral episomal vectors also include mammalian artificial chromosomes (MACs), which can be generated through both top-down and bottom-up approaches [57,58]. However, current progress with MACs is limited because of prohibiting costs associated with the generation of these vectors.

4.7. Employment of the locus control regions within the vectors

Protection of integrated transgenes from encroaching heterochromatin can be achieved with chromatin insulators or other *cis*-acting locus control regions (LCRs) [59]. The mechanistic details of LCRs action are currently not clear and so the terminology in this area is somewhat diffuse with, for example, 'chromatin boundary elements' and 'chromatin insulators' often being used synonymously [60,61]. Some enhancers have an important bearing on the state of chromatin and, therefore, can also be viewed as LCRs. Experiments with some known chromatin insulators show that their effects on transgene expression are not always positive and to a large extent depend on the cell context [62,63]. Nuclear 'matrix attachment region' elements (MARs) and the effectively synonymous nuclear 'scaffold attachment region' elements (SARs) are known to possess some LCR activity. Some authors are trying to avoid the confusion between MARs and SARs using the joined names 'SAR/MARs', 'MAR/SARs' or 'S/MARs'. Promising results in terms of sustained transgene expression were achieved with MARs both within the scenario where two MAR elements are used 'to protect the transgene from the flanks' [64,65] and the scenario where a single promoter-MAR couple is driving the transgene's episomal replication [41].

4.8. Top-up transgene administration to compensate for silenced transgenes

Normally, if the expression of therapeutic transgenes did die out, it is possible to perform a new round of gene transfer, thus achieving a new burst of transgene expression. Repeated vector administration can be particularly sought-after when the target cell population experiences programmed death, while the respective progenitor cell pool is poorly accessible for therapeutic gene transfer. This strategy can be used without hesitation in an *ex vivo* gene therapy setting where therapeutic genes are delivered *in vitro* to dividing cells derived from a patient's biopsy prior to autologous transplantation. In contrast, in an *in vivo* gene therapy setting, the drawbacks of vector re-administration include not only the increased complexity and cost of treatment, but also the realistic possibilities that immunity to elements of the vector might develop and that the effects of the toxic elements of the vector might build up to an unacceptable level. That is why low immunogenicity, low toxicity and the biodegradability of auxiliary vector elements are important in the vector re-administration treatment format.

4.9. Selection of clones with stable non-silenced transgene expression

Reliable, robust and error-free site-specific integration into mammalian cells lacking pre-engineered integration sites is difficult to achieve. Simpler alternatives for attaining stable long-term transgene expression exist in the *ex vivo* gene therapy approach. In one of the treatment scenarios, transgenes are integrated randomly, e.g. using lentiviral vectors or naked DNA vectors. It is then possible to select the best clone with minimal initial transgene silencing and minimal propensity for transgene expression shutdown among a heterogeneous population of transfected or transduced cells. The preferred method for cell selection is antibody-based magnetic sorting, as this method allows processing of large numbers of cells without recourse to heterologous fluorescent proteins and mutagenic UV irradiation as in fluorescence activated cell sorting (FACS). Clearly, such a clone pre-selection strategy can be used in conjunction with some other counter-silencing strategies (e.g. multiple random transgene insertions or top-up transgene administrations).

4.10. Small molecule enhancers of transgene expression

It is extremely attractive to use small molecule compounds to counteract transgene silencing. Substances known to influence chromatin's state are prime candidates for this role. Thus, histone deacetylase inhibitors Trichostatin A, 4-phenylbutyric acid, butyric acid, valeric acid and caproic acid were successfully used to enhance transgene expression after transient transfection [66]. Available data indicate that another histone deacetylase inhibitor, valproic acid, and also retinoic acid, which is known to act through a receptor-mediated mechanism, are epigenetically active substances and, therefore, in certain situations could be considered for use as transgene expression stimulants. Some small molecule enhancers could be specific for particular vectors used for gene transfer. Thus, hydroxyurea is known to boost transgene expression after delivery with AAV vectors [67]. In this case, transgene expression is likely to be spurred not through the inhibition of standard silencing mechanisms but rather

through the more active synthesis of the second DNA strand in the delivered single-stranded AAV vector DNA [67].

4.11. Selection of low immunogenic vectors and transgene products

The elimination of therapeutic gene vectors and transgenic cells by the immune system can imitate the silencing of transgene expression. Thus, the employment of low-immunogenic vectors is a preferred option. Vectors' epitopes should mimic the native epitopes of individual patients and do not match their pre-existing immune profile. Coating vector particles with immunologically inert polymers like polyethyleneglycol is one of the strategies to escape immune surveillance. Alternatively, vector particles can be developed, which are able to mimic the immune-evasion strategy of some viruses that are capable of 'hiding' at the cell surface [68]. Non-immunogenic transgene products, e.g. exclusively human versions of proteins, should be chosen to prevent cell elimination via immune reactions *in vivo*. If required, transgene products should be re-engineered to achieve the 'stealth effect' and to tailor them to the immunological profiles of individual patients.

5. Conclusion

Epigenetic control by the target cells can result in permanent transgene silencing or in the instability of transgene expression. Thus, one needs to pursue therapeutic strategies, which can achieve long-term transgene expression by taking advantage of, circumventing or overriding silencing favouritism of the resident gene expression control mechanisms.

There are many levels at which the longevity of transgene expression can be addressed through the gene vector choice, design and administration regimen, including: 1) employment of non-nuclear vectors, e.g. mRNA or Sendai virus based vectors; 2) control of transgene modules' amenity to methylation (e.g. purposeful exclusion of methylation-prone CpG islands); 3) employment of minimised DNA vectors such as minicircle DNA to avoid transgene silencing by the bacterial portion of the plasmid vectors; 4) choice of a suitable promoter-enhancer combination with the judicious use of tissue specific, inducible and ubiquitous promoters; 5) achieving a high number of randomly integrated transgenes; 6) control of the chromosomal integration sites via artificial site-preferences of retroviral integrases, transposases or via harnessing of site-specific integration systems; 7) localisation of transgenes on nuclear episomes; 8) chromatin re-modelling control via *cis*-acting elements such as insulator elements and other LCRs; 9) repeated vector administration; 10) selection of individual cell clones with transgenes integrated into favourable loci; 11) use of chemical reagents influencing the epigenetic state to achieve higher and more long-term transgene expression; and 12) choice of non-immunogenic transgene products to prevent the elimination of transgenic cells via immune reactions *in vivo*.

Clearly, the future solutions to transgene silencing enabling stable long-term expression of therapeutic transgenes will depend on the determined implementation of the above strategies and their effective combinations.

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Gene Therapy Tools: Synthetic

Cellular Uptake Mechanism of Non-Viral Gene Delivery and Means for Improving Transfection Efficiency

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Additional information is available at the end of the chapter

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1. Introduction

Non-viral delivery systems usually include mechanical, electrical, and chemical methods. Cationic liposomes and cationic polymers are two typical classes of non-viral vectors. Compared with viral vectors, non-viral ones are considered promising vehicles for gene therapy because of their low toxicity, biocompatibility, and controllability [1, 2], although their low efficacy limits their application as a mature gene delivery system. Improving the efficacy of non-viral vectors necessitates thorough understanding of their *in vivo* key steps. Non-viral vectors can complex with gene materials and help them access the target compartments within cells. Many barriers prevent gene materials from reaching their intended target and performing their functions [3], safe and effective delivery remains an important challenge for the clinical development of non-viral vectors [4].

The delivery of pDNA or siRNA *in vivo* for therapeutic aims has been widely studied in recent years. However, non-viral delivery systems, which exhibit relatively low levels of efficiency, are not clinically applicable. Improving their efficiency is the main task of pDNA- or siRNA-based gene therapy. There are many barriers that hinder pDNA and siRNA from reaching their intended target in the plasma and performing their functions: First, gene materials can be loaded into vectors. After *in vivo* administration, the vectors must be delivered to the blood vessels and should be stable in the blood; otherwise, they will be cleared by albumin because of their high surface charge and may also be uptaken by macrophages. The vectors must then pass through the epithelial tissue of the blood vessels and enter the target tissue. As it is very difficult for nanoparticles larger than 5 nm in diameter to pass through the epithelial tissue of blood vessels [5], it is crucial to study the cellular transport mechanism of epithelial cells through the caveolin-mediated endocytosis (CvME) pathway which is active in epithelial cells [6]. The distance between the extracellular matrix and target cells

is great, and many vectors will be uptaken and cleared by macrophages after they do manage to pass through the epithelial tissue of blood vessels.

Next, the vectors must attach to the cell membrane, which entails other issues altogether. First, the non-viral vectors should be able to identify specific cell types to ensure safety. They then enter cells mainly via endocytosis. Different endocytosis pathways yield different intracellular fates for vectors, which could potentially explain why the same vector differs in its transfection efficiency in various cell modes. After their entry into the cells, vectors must escape from the endosome or avoid the endo-lysosomal (endosomal and lysosomal) pathway through certain endocytosis pathways. After escaping the endosome and then entering the cytoplasm, vectors must release pDNA or siRNA and finally perform their function in the cytoplasm [5]. In addition, pDNA has to be transported into the nucleus. The key steps in non-viral delivery are shown in Figure 1.

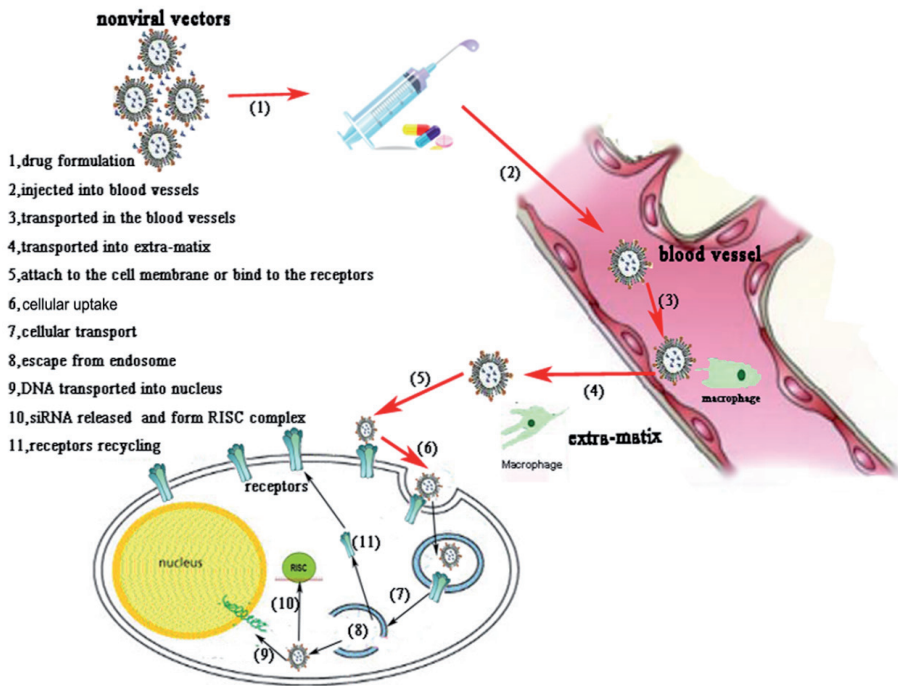


Figure 1. Biological key steps of non-viral vectors

As discussed above, the cellular process (including uptake, transport, endosomal escape, and nuclear localization) is one of the most important steps for non-viral gene delivery. In 2001, Hideyoshi Harashima et al. stated that novel strategies of medical treatments, such as

gene therapy, highlight the importance of studying the intracellular fate of macromolecules, such as DNA and siRNA. In particular, in the case of gene therapy, intracellular events would be expected to be the major factors controlling the fate of the introduced gene and the efficiency of its expression. These authors attempted to establish an intracellular pharmacokinetic model of genes to study the intracellular events involved in gene therapy [7]. Understanding the intracellular fate of a gene or vector is important for us to overcome the cellular barriers of DNA or siRNA delivery and rationally design efficient systems thereof.

Of all intracellular events, the cellular uptake mechanism of non-viral vectors is the most essential to their efficiency and intracellular fate. Different cellular uptake pathways have different intracellular fates. As the gene materials will be degraded in the endo-lysosomes (endosomes and lysosomes). One good example is that some endocytic pathways involve endo-lysosomes, but others that can bypass the endo-lysosomes have higher levels of delivery efficiency. Polyethylenimine (PEI) is one of the most promising non-viral vectors [8]. Some researchers have shown that cellular uptake of PEI polyplexes affects other cellular processes and, consequently, transfection efficiency [9, 10]. These differences may depend on such factors as the size, surface properties, and shape of the particles [11], as well as different cell lines [9].

Research has shown that polyplexes and lipoplexes have different uptake mechanisms in A549 pneumocytes and HeLa cells. Lipoplex uptake proceeds only by clathrin-mediated endocytosis (CME), whereas polyplexes are taken up by two mechanisms — one involving caveolae and another using clathrin-coated pits [10]. As the caveolae-mediated uptake mechanism has slower kinetics, the transfection process of polyplexes is slower than that of lipoplexes in A549 pneumocytes and HeLa cells. However, as the polyplexes uptaken via the caveolae escape the lysosomal compartment, polyplexes have a high level of transfection efficiency [10]. Taken together, these findings highlight the importance of studying the cellular uptake of non-viral vectors, their intracellular fate, and their effects on transfection efficiency. Understanding cellular uptake mechanisms is crucial to engineering successful reagents or vectors for non-viral gene transfection [12].

2. Cellular uptake pathways of non-viral gene delivery

The uptake pathways are divided into two groups: endocytic pathways and non-endocytic pathways. Inside endocytic group, there are two types of pathways: phagocytosis and non-phagocytosis pathways [11].

2.1. Phagocytosis

Phagocytosis is a special type of endocytic pathway which primarily exists in professional phagocytes such as macrophages, monocytes neutrophils, and dendritic cells (DCs) [13]. In comparison, other nonphagocytic pathways such as clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis occur in almost all kinds of cell types [14]. Phagocytic pathway is mediated by cup-like membrane extensions that are

usually larger than 1 μm to internalize large particles such as bacteria or dead cells. Understanding of the mechanism of phagocytosis is very helpful to the non-viral gene therapy of macrophage-dominated immune diseases such as rheumatoid arthritis. In addition, a phagocytosis-like mechanism was proposed for the uptake of large lipoplexes and polyplexes that are larger than can be taken up by the classic CME pathway [15, 16].

Phagocytosis depending on opsonins can be called as opsonic phagocytosis. There is also another phagocytosis which is opsonins independent. This will be discussed later. First, for opsonic phagocytosis, the complexes will be recognized by opsonins in the bloodstream. Then, the opsonized complexes adhere to professional phagocytes and are ultimately ingested by them [11]. Opsonization is the key step of the phagocytosis pathway. It involves complexes tagged by some major opsonins including immunoglobulins G and M (IgG and IgM), as well as complement components C3, C4, and C5 in the bloodstream [11, 17]. These opsonized complexes become visible to macrophages and bind to their surface through the interaction between receptors (such as fragment crystallizable receptors (FcR) and complement receptors (CR)) and the constant fragment of particle-adsorbed immunoglobulins.

Other receptors that mediate phagocytosis pathway have also been reported. Mannose receptor (MR) has been used in gene vaccine by targeting human DCs and macrophages through the phagocytic pathway [18]. Scavenger receptor (SR)-mediated delivery of anti-sense minixen phosphorothioate oligonucleotide to leishmania-infected macrophages is proved to be selective and efficient in eliminating the parasite [19]. SR-A, macrophage receptor, and CD36 are the three SR subtypes. CD36 can mediate non-opsonic phagocytosis of pathogenic microbes [20]. Unlike opsonic phagocytosis, non-opsonic phagocytosis is directly mediated by the receptors on the cell surface without the help of opsonins. This kind of mechanism can also be used for gene delivery.

Then the activated Rho-family GTPases trigger actin assembly and cell surface extension formation. This surface extension finally zippers up around the complexes and engulfs them [11]. The phagosomes carrying the complexes fuse with lysosomes to form mature phagolysosomes [11]. In phagolysosomes, the complexes undergo a process of acidification and enzymatic reaction. As the intracellular fate of phagocytosis is the transportation of complexes into the lysosome, the gene materials will be degraded by the nucleases inside it [21]. Endosomes and lysosomes (endo-lysosomes) are very important biological barrier for gene delivery. The vectors should have capability to escape from them, if gene materials loaded vectors entry into the cell via phagocytosis. The mechanisms of endo-lysosome escape will be discussed later.

2.2. Non- phagocytosis pathways

Non- phagocytosis pathways mainly include clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis. CME is the best-characterized type of endocytosis, which is receptor-dependent, clathrin-mediated, and GTPase dynamin-required [22, 23]. The uptake of low-density lipoprotein and transferrin is typically via this endocytic pathway, and they are often used as the CME probes in many studies [24, 25]. Transferrin has also been used as a ligand of non-viral vectors to improve the endocytosis of

complexes [26, 27]. In this pathway, a series of downstream events are activated after the recognition of ligands by receptors on the cell surface. Clathrins assemble in the polyhedral lattice right on the cytosolic surface of the cell membrane, which helps to deform the membrane into a coated pit with a size about 100–150 nm [27]. This process is mediated by GTPase dynamin. As the clathrin lattice formation continues, the pit becomes deeply invaginated until the vesicle fission occurs. In the next step of the CME pathway, the endocytosed vesicles internalized from the plasma membrane are integrated into late endosomes and finally transported to lysosomes.

CvME begins in a special flask-shaped structure on the cell membrane called caveola, which is a kind of cholesterol- and sphingolipid-rich smooth invagination [28]. CvME usually happens in the vessel wall lining monolayer of endothelial cells [7]. Caveolae have a diameter range of 50–100 nm [11] and are typically between 50 and 80 nm with a neck of 10–50 nm [6]. CvME is also a type of cholesterol, dynamin-dependent, and receptor-mediated pathway [29]. The fission of the caveolae from the membrane is mediated by the GTPase dynamin, which locates in the neck of caveolae and then generates the cytosolic caveolar vesicle [11]. Some receptors located in caveolae, such as insulin receptors [30] and epidermal growth factor receptor (a type of receptor in ovarian cancer) [31], can mediate CvME [32]. The vesicle budding from the caveolae, a type of caveolin-1-containing endosome is called caveosome [29]. The intracellular fate of the caveosome differs from that of CME. Compared with CME, CvME is generally considered as an alternative pathway which can deliver the vectors into Golgi and/or endoplasmic reticulum, thus avoiding the normal lysosomal degradation.

Macropinocytosis is a type of distinct pathway that nonspecifically takes up a large amount of fluid-phase contents through the mode called fluid-phase endocytosis (FPE) [33]. Macropinocytosis is a signal dependent process that normally occurs when macrophages or cancer cells are in response to colony-stimulating factor-1 (CSF-1), epidermal growth factor (EGF) and platelet-derived growth factor or tumor-promoting factor, such as phorbol myristate acetate respectively [34–36]. However, this process occurs constitutively in antigen-presenting cells [37]. Macropinocytosis occurs via the formation of actin-driven membrane protrusions, which is similar to phagocytosis. However, in this case, the protrusions do not zipper up the ligand-coated particle; instead, they collapse onto and fuse with the plasma membrane [11]. The macropinosomes have no apparent coat structures and are heterogeneous in size, but are generally considered larger than 0.2 μm in diameter [38, 39]. During this process, the small GTPase, Ras-related in brain (Rab) proteins are essential for the vesicle fission from the cell membrane [40]. The relationship between the macropinocytosis and lysosome is still unknown. This will be discussed later.

2.3. Non-endocytic pathways

There are three technologies that are designed to mediate the non-endocytic pathways and successfully transfect the gene. One is microinjection, by which each cell is injected with the gene materials using glass capillary pipettes. The second one is permeabilization by using pore-forming reagents such as streptolysin O or anionic peptides such as HA2 subunit of the influenza virus hemagglutinin. The third one is electroporation,

which uses an electric field to open pores in the cell. All of them are highly invasive and not ideal for in vivo gene delivery.

However, There are evidences which can prove the existing of other non-endocytic pathways. One pathway is related to the formation of holes in the cell membrane, called “penetration”. A class of cationic peptides with the protein transduction domains (PTDs), such as TAT, has the ability to be taken up without endocytic events [41]. These peptides can directly penetrate cell membranes in a receptor-, and energy-independent way. In 2004, Hong et al. studied the hole formation on the cell membrane induced by poly(amidoamine) (PAMAM). The results indicated that the hole formation can be induced by positively charged PAMAM, and labeled PAMAM can diffuse into the cells through small holes in the membrane. This mechanism is considered a nonspecific pathway, which is not receptor-mediated and lacks selective cellular uptake [42]. In 2010, Lee et al. used a PTD called Hph-1 to conjugate vector PEI to deliver siRNA. The result showed that the complexes entered the cells through the non-endocytic pathway, which has a quicker dynamic behavior compared with the endocytosis pathways and is energy-independent because it has high transfection efficiency even in low temperature [43]. Another non-endocytic pathway is called “fusion”, which is special for lipoplexes, as it can cause a direct release of DNA to the cytoplasm before entering the endocytic pathways. However, more and more evidences suggest that fusion with the cell membrane contributes minimally to the overall uptake of lipoplexes, while the CME plays an important role in the uptake of lipoplexes [44]. there have been few studies on non-endocytic pathways, and more efforts are needed to have a comprehensive understanding of these pathways for the improvement of non-viral gene delivery.

Pathways	GTPases	Relationship with lysosome	Receptors
Phagocytosis	Rho	Dependent	Dependent
CME	Dynamin	Dependent	Dependent
CvME	Dynamin	Independent	Dependent
Macropinocytosis	Rab	In dispute	Non-specific
Non-endocytic	Independent	Independent	Non-specific

Table 1. Cellular uptake mechanisms.

3. Factors that influence the uptake pathways of non-viral gene delivery

There are many factors that are involved in the selection of uptake pathways of non-viral gene complexes. These factors include particle size, particle surface charge, particle shape, cell type, and even culture condition. Because the complexes of non-viral gene vector/DNA or siRNA are usually a group of heterogeneous particles with diverse sizes, surface charges, and shapes, several uptake pathways may be involved in the internalization of one kind of

complexes into a single cell type. For example, transfection by branched PEI25kDa/DNA polyplexes was mediated by both CME and CvME pathways in HUH-7 and Hela cells [9]. Later, Hansjörg Hufnagel reported that the macropinocytosis is also very important for the uptake of branched PEI25kDa/DNA polyplexes into Hela and CHO-1 cells due to the existence of large particles of polyplexes (>500 nm) [12]. Therefore, the heterogeneity of complexes has to be taken into consideration when the results are analyzed. Particle size is a very important factor for the pathway selection of complexes. As mentioned above, the labeled cationic PAMAM can induce hole formation in the cell membrane. The holes induced by PAMAM are 15–40 nm in diameter [42]. The particle including the gene complex, which is smaller than these holes, can diffuse through the holes and be taken up by nonspecific non-endocytic rather than specific receptor mediated endocytic pathways. PEI/DNA complexes with sizes smaller than 500 nm are mainly taken up by CME and CvME according to a previous study [10]. While PEI/DNA complexes with sizes >500 nm are mainly internalized by macropinocytosis pathway [10].

The charge density of a complex is also an important factor for its uptake. The cell membrane consists of a bilayer of lipid and anionic membrane proteins. These anionic proteins are very helpful to the uptake of cationic complexes. However, once the net positive charge falls to neutral, the uptake efficiency will be inhibited a lot. This is because the neutral charge density will weaken the interaction between complexes and membrane proteins, and it will also increase the aggregation of complexes, which will make them large and hard to be internalized. This change can be caused by the anionic proteins in the *in vivo* circulation of blood, and the serum used in the *in vitro* transfection medium. The modification of polyethylene glycol (PEG) can solve this problem with its high hydrophilicity, electrical neutrality and steric-repulsive propensity [45].

As to the relationship between the shape of particles and the pathway selection, few studies have been made about this issue. A group once reported that the uptake of protein-coated spherical gold nanoparticles is more efficient than rod-shaped ones in Hela cells, SNB19 cells, and STO cells [46, 47]. However, as to the relationship of nonviral gene complexes and their uptake efficiency, it is not easy to draw such a conclusion, because the non-viral gene complexes are usually a group of nanoparticles with heterogeneous shapes, and their shapes are dependent on the experimental conditions. Taking chitosan as an example, the fraction of complexes that have nonaggregated, globular structures increases with increasing chain length of the chitosan oligomer, increasing charge ratio and reduction of pH (from 6.5 to 3.5) [48]. Because of this, this complicated issue leaves much room for researchers to discuss.

Cell type is another important factor that influences the pathway selection of non-viral gene complexes. Different types of cells can take up a kind of complex in different pathways. Most of the studies focused on COS-7 cells, which were used as a well-established model cell for gene delivery researches [28]. Some researchers also used other cell lines such as A549, Hela, and HUH-7 cells. Caveolae, which are a very important structure for CvME pathway, are present in many cell types, but they are particularly abundant in the vessel wall lining monolayer of endothelial cells. As a result, endothelial cells have been especially used in studies on CvME pathway. A study tested the endocytosis pathways involved in the

transfection of PEI/DNA complexes with different cell lines. The result showed that in COS-7 cells, the clathrin-dependent pathway was the main contributor to the transfection process for both linear and branch PEIs [9]. Another study suggests that macropinosomes have a higher propensity to deliver PEI/DNA cargo than do endosomes in CHO and Hela cells [12]. Therefore, different cell lines involve different endocytic pathways, and cell type is the important factor that must be considered in such studies.

4. Tools for the study of uptake pathways

The study on the mechanisms of uptake pathways is important to the rational design of non-viral gene vectors because this step can determine the intracellular fate of complexes. However, because there are many factors that influence the pathway selection, how to conduct these studies is also a very complicated problem that needs to be discussed in detail.

Inhibitors are the effective tools to block specific pathway in order to determine whether it plays an important role in the uptake of complexes. However, none of the commonly used inhibitors of different uptake pathways is absolutely specific. All of them either affect the actin cytoskeleton with their side effects, or interfere with alternative uptake pathways simultaneously. In addition, they usually show cell type variations. The scope of the usage of commonly used inhibitors will be introduced according to the classification of uptake pathways in the following paragraphs of this section. The most direct way to distinguish endocytic pathways and non-endocytic pathways is to use the inhibitor or method of energy depletion, because most endocytic pathways are energy dependent. The commonly used inhibitors and methods are: low temperature (4 °C) and sodium azide (an ATPase inhibitor). Low temperature and ATP inhibitor should be used together in some conditions because some of the non-endocytic pathways are also sensitive to low temperature [42, 49].

To distinguish the phagocytic and macropinocytic pathways with CME and CvME pathways, the commonly used inhibitors and methods for phagocytic and macropinocytic pathways are: inhibitors of sodium-proton exchange “amiloride and its derivatives”, F-actin depolymerizing drugs “cytochalasin D and latrunculin”, inhibitors of phosphoinositide metabolism “wortmannin and LY290042”, and protein kinase C activator “phorbol esters”. Except phorbol esters, the specificity of all the inhibitors is still in doubt as depolymerizing F-actin and inhibition of phosphoinositide metabolism may also disrupt the other two endocytic pathways. For example, cytochalasin D is also used as the inhibitor for CvME [50]. Within these inhibitors, amiloride and its derivatives may be considered as the first choice for their fewest side effects. Rottlerin, a novel macropinocytosis inhibitor which is rapid acting, irreversible, and selective, was discovered in 2005. In 2009, Hufnagel et al. found that rottlerin can specifically inhibit the transfection efficiency of PEI (25 kDa)/DNA complexes on Hela and CHO-K1 cells up to 50%, which verified the important role of FPE in the non-viral gene delivery by PEI (25 kDa) [12].

The commonly used inhibitors and methods for clathrin-mediated endocytosis are: Hyper-tonic sucrose (0.4–0.5 M), potassium depletion, cytosolic acidification, chlorpromazine (50–

100 μ M), monodansylcadaverine (MDC), phenylarsine oxide. However, all of them have been shown to be able to inhibit macropinocytosis, thus cannot be used to distinguish the clathrin-mediated endocytic pathway and the macropinocytic pathway. Besides this, all these inhibitors can influence the cortical actin cytoskeleton more or less, which can cause non-specific cytotoxicity. However, potassium depletion, chlorpromazine, and MDC are the relatively better choices than the other ones for the initial discrimination of clathrin-mediated endocytic pathway [51].

As to caveolae-mediated endocytic pathway, the commonly used inhibitors and methods are: statins, methyl- β -cyclodextrin (M β CD), filipin, nystatin, genestein, and cholesterol oxidase. Among them, the incubation with filipin, nystatin, and cholesterol oxidase produce the fewest side effects. The chronic inhibition of cholesterol synthesis by statins or acute cholesterol depletion by M β CD nonspecifically disrupts intracellular vesicle trafficking and the actin cytoskeleton. Also, the specificity of genestein is still in doubt for its nonspecific disruption of the actin network. That being so, appropriate controls should be included when filipin, nystatin, and cholesterol oxidase are used [51].

The inhibitors for the study of intracellular fates of complexes are also very important. Monensin, bafilomycin A can inhibit the acidification of endosomes, thus preventing their maturation and fusion into lysosomes [52, 53]. Chloroquine is another inhibitor that accumulates in endosomes/lysosomes and causes the swelling and disruption of endocytic vesicles by osmotic effects [21]. Last but not least, the cell-dependence of inhibitors should be noted when experiments are carried out. For example, chlorpromazine treatment inhibited the uptake of transferrin, a marker for CME by ~50% in D407 and HUH-7 cells. However, it showed no or little significant inhibitory capacity in ARPE-19 and Vero cells or even an enhanced effect in COS-7 cells [54, 55]. Therefore, a range of concentration with lowest cytotoxicity and sufficient inhibitory efficiency should be determined first when the inhibitor is used on the cell for the first time. Then, the lack of absolute specificity can be compensated by the combined application of biological methods such as siRNA silencing, transient or stable expression of dominant-negative proteins, and reconstruction of proteins by knockout mutants, all of which are more specific than classical chemical inhibitors. For example, mutant dynamin has been successfully used to prove the necessity of dynamin in the endocytic pathways of transferrin receptors and EGF receptors [55]. A constitutive knockdown technique through RNAi has been used to prove the role of an essential accessory protein “epsin” in the CME pathway [56]. Another efficient way of making up the pitfalls of nonspecific inhibitors is the combined usage of fluorescently labeled gene complexes and fluorescent probes that are specifically internalized through certain uptake pathways.

Except for inhibitors, molecular probes and markers are also important tools for the study of uptake pathways for non-viral gene complexes. They can be used together with the classical chemical inhibitors or biological inhibitors to make the results more convincing. There are several classical molecular probes that are known to be specifically internalized through each uptake pathway. Transferrin is often used as a probe of CME pathway in many studies [12, 57, 58]. Transferrin receptor (TFR) mediates transferrin uptake by CME, so that it can be used as a CME marker and detected by anti-TFR [59].

Cholera toxin beta subunit (CTBs) is commonly used as a probe for CvME [12, 57]. However, Lisa et al. argued that CTBs binds receptors that are contained in lipid-rich areas and are internalized via a mechanism similar to CvME, because CTBs uptake is unaffected by a clathrin inhibitor and 33% uptake remains after treatment with a specific caveola inhibitor. Therefore, CTBs may enter into the cells via another unknown clathrin-independent mechanism [60]. In addition, caveolin-1 is also an important marker for CvME, as it is specifically involved in the formation of caveosome [29].

Dextran is the popular probe for macropinocytosis in some studies because it can accumulate in the endo-lysosome compartment [57]. As to phagocytosis, large (2 μ m) microspheres are usually used as the probes. To solve the issue about the intracellular fate of complexes, a group of the specific markers or biological dyes are necessary to colocalize the non-viral gene complexes and intracellular organelles. TFR is used as a classical early endosome marker because it is transported into an early endosome when transferrin is internalized. EEA-1 is a hydrophilic peripheral membrane protein present in cytosol and membrane fractions. It colocalizes with TFR, and immunoelectron microscopy shows that it is associated with tubulovesicular early endosomes [61]. The lysosome-associated type 1 membrane glycoproteins LAMP-1 and LAMP-2 are localized primarily on the periphery of the lysosome, and can be used as markers for lysosome [62, 63]. The different roles of EEA-1 and LAMP in the endolysosome pathway allow us to know the stage in which the uptake carries on. Other endosome or lysosome markers are the Rab family proteins. They are small GTPases that control multiple membrane trafficking events in the cell, and there are at least 60 Rab genes in the human genome [64]. Inside the Rab family, Rab5 and Rab7 are the most studied Rab variants, in which Rab5 is found to be the marker for early endosomes as it in part controls the invagination at the plasma membrane, endosomal fusion, motility, and signaling [63], and Rab7 is found to be the marker for late endosomes and lysosomes as it controls the aggregation, fusion, and maintenance of perinuclear lysosome compartment [65].

Pathways	inhibitors	markers
Phagocytosis	Amiloride, cytochalasin D, latrunculins, wortmannin, LY290042, sodium azide	Large microspheres (2 μ m)
CME	Chlorpromazine, monodansylcadaverine, phenylarsine oxide, sodium azide	Transferrin, lactosylceramide, TFR
CvME	Filipin, nystatin, cholesterol oxidase, statins, genestein, M β CD, sodium azide	CTBs, caveolin-1
Macropinocytosis	Rottlerin, amiloride, cytochalasin D, latrunculins, wortmannin, LY290042, sodium azide	Dextran

Table 2. Inhibitors and markers

The organelle specific dyes are other ideal tools for the detection of colocalization, and they are relatively convenient. LysoTracker (red) and Lyso Sensor (green) are the widely used

dyes for lysosomes. Cell light (red or green) are the widely used dyes for early endosomes. Combined with the confocal imaging technology, the colocalization of labeled non-viral gene complexes and intracellular compartments can be viewed intuitively. However, the classical confocal imaging technology can only provide the monolayer images, the information from which is not convincing enough. A novel three dimensionally integrated confocal technology is so strong that it can provide the intact information of a whole cell by scanning layer by layer.

5. Application of cellular uptake mechanism.

Based on the current understanding of cellular uptake mechanisms, one can rationally design vectors and improve their efficiency. Each pathway has advantages that need to be optimized and disadvantages that should be avoided (Table 3).

Pathways	Advantages	Disadvantages
Phagocytosis	Specific cell-type targeting Specific receptors	Lysosome involved In vivo clearance
CME	Specific receptors	Lysosome involved
CvME	Bypass the lysosome Specific receptors	Membrane structure dependent Slower cellular uptaking
Macropinocytosis	Larger particles uptaking.	Non-specific
Non-endocytic	Bypass the lysosome	Non-specific

Table 3. Characteristics of pathways.

5.1. Endo-lysosomal escape

Endo-lysosomal escape is one of the most crucial issues in non-viral vector design. Non-viral delivery systems, such as polyplexes and lipoplexes, will be trapped and degraded in the lysosomes if their cellular uptake pathways involve endo-lysosomes. As discussed above, some of the uptake pathways involve endo-lysosomes, such as CME and phagocytosis. CvME is known to bypass the endo-lysosomes. Similarly, macropinocytosis is known to not have any associations with endo-lysosomes [66, 67], but some studies have suggested that it involves lysosomes [67, 68]. These contradictory data may be dependent on cell type. Stimulating special pathway to bypass endo-lysosomes is a novel direction to improve efficiency. This will be discussed later.

A non-viral delivery system uptaken by endo-lysosomes dependent pathways must be capable of escaping endo-lysosomes. From early endosome to late endosome transport, a maturation process involving compartment acidification by proton pumps located on the endosomal membrane exists. Some non-viral vectors exhibit the ability to escape the endo-

lysosome, called *proton sponge*, such as PEI [10, 69, 70]. PEI contains a nitrogen atom that can be protonated, and this serves to consume endosomal protons because endosomes acidify their microenvironment. As a result, an increase in endosomal chloride anion, which diffuses into the endosomes with the protons, leads to an increase in osmotic pressure, thus inducing osmotic swelling [69]. Therefore, the endosome might break down and release PEI. This mode of action has been widely incorporated in recent non-viral vector designs. However, a pDAMA-based vector with endosomal buffering capacity has been reported to show no endosomal escape activity in cell-based assay, indicating that the proton sponge hypothesis may not be applicable in some cases. These findings warrant further elucidation and investigation of the mechanism of non-viral gene delivery [71].

For lipoplexes, the cationic liposome can interact with the anionic cytoplasmic facing monolayer lipid of endosome and release the DNA from the endosome through the flip-flop mechanism [72]. 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), the pH-sensitive fusogenic lipid additive, is very helpful to the displacement of the anionic lipids from the cytoplasm-facing monolayer of the endosomal membrane to the opposite direction via a flip-flop mechanism. However, the serum components are known to inactivate and destabilize the lipoplex structures that contain DOPE [73].

Viruses have the ability to destabilize the endosomal membrane, which explains why many proteins from different viruses are being used [69]. Some viruses are well known to use fusogenic peptides to cross the endosomal membrane and reach the cytosol [21]. The process by which viruses destabilize endosomal membranes in an acidification-dependent manner has been mimicked with synthetic peptides containing the amino-terminal 20-amino-acid sequence of the influenza virus HA [70]. Generally, short sequences of only 20 amino acids are needed for membrane destabilization, and they usually contain a high content of basic residues [74].

Cell-penetrating peptides (CPPs) are used to enhance endosomal escape. The HIV-1 Tat protein is the first CPP to be discovered. It transactivates the transcription of the HIV-1 genome, has been observed to cross the plasma membrane by itself, leading to the identification of a peptide fragment (49–59 amino acids) that confers cell permeability to the protein (Tat peptide), and is one of the better characterized CPPs [75]. Most of the CPPs contain a high density of basic amino acids (arginines and/or lysines), which are proposed to interact with the anionic surface of the plasma membrane and enhance internalization of the peptides [75]. These peptides adopt an α -helical structure at endosomal pH leading to hydrophobic and hydrophilic faces that can interact with the endosomal membrane to cause disruption and pore formation [74].

5.2. Optimization of CvME

CvME is considered an alternative pathway that can bypass the endo-lysosomes. As gene materials will not be degraded in the lysosomal compartments, we can take advantage of CvME to improve the efficiency of transfection. For example, Nathan P. et al. targeted complexes (PEI-DNA) in CvME and CME with folic acid and transferrin, respectively; however, only vectors via CvME successfully delivered genes, as CvME is avoidant of lysosomes.

These data demonstrate that the uptake mechanism and subsequent endocytic processing are important design parameters for gene delivery materials [76]. However, the key is controlling the uptake mechanism.

Particle size is a very important factor for uptake mechanisms. In a previous study, three particles (20, 40, and 100 nm) were investigated for their uptake efficiency via CvME in endothelial cells. The results showed that the uptake efficiency levels of the 20- and 40-nm nanoparticles were 5–10 times greater than that of the 100-nm particles [6], indicating that small particles can be uptaken by CvME more efficiently compared with large ones. However, another study found that the uptake of microspheres with a diameter <200 nm in non-phagocytic B16 cells involved CME. With increasing size, a shift to a mechanism that relied on a caveolae-mediated pathway became apparent, which became the predominant pathway of entry for particles measuring 500 nm in size [77]. This can be attributed to the fact that the mechanism of CvME is cell type dependent in some cases. According to the target cell type, the mechanism must be fully studied before designing a vector.

CvME is a kind of receptor-mediated endocytosis pathway. As a result, some specific ligands can mediate CvME via ligand–receptor binding. The insulin receptor [30], epidermal growth factor (EGF) receptor [31], transforming growth factor beta (TGFβ) receptor [78] have been found to mediate this pathway. Another study used the cyclic Asn–Gly–Arg peptide to enhance gene transfection efficiency in CD13-positive vascular endothelial cells via CvME [79]. However, cyclic RGD ligands have been reported to facilitate CvME of thiolated c(RGDfK)-polyethylene glycol (PEG)-b-PLL micelles without high endosomal-disrupting properties and thus improve transfection efficiency [80]. The cyclic RGD peptide ligands c(RGDfK) can selectively recognize αvβ3 and αvβ5 integrin receptors on the cell surface. The receptors can mediate CvME and bypass endo-lysosomes. The αvβ3 and αvβ5 integrin receptors overexpressed on endothelial cells of tumor capillaries and neointimal tissues. As a result, the vectors with cyclic RGD peptide ligands can be used for cancer gene therapy.

Cellular stress can also be used to control the cellular uptake mechanism. Heat shock and hyperosmotic shock can stimulate caveolin internalization [81]. Recent research has shown that hypertonic exposure of alveolar cells caused down-regulation of CME and fluid-phase endocytosis while stimulating CvME. An osmotic polymannitol-based gene transporter that can increase caveolae-mediated endocytosis was designed taking advantage of this mechanism [82]. The possible mechanisms have been discussed. Non-penetrating osmolytes tend to draw water from the intracellular space through an osmotic gradient, cause cell hypertonic stress accompanied by cell shrinkage. Responding the cellular hypertonic stress, phosphorylation of caveolin-1 is mediated by Src-kinase. Src-kinase-mediated phosphorylation of caveolin-1 is required for caveolae budding. Finally the CvME is stimulated.

5.3. Inhibition of phagocytosis

After *in vivo* administration, the non-viral delivery system can be uptaken by macrophages and then cleared. This macrophage clearance effect mainly via phagocytosis is one of the main barriers for non-viral gene delivery. Numerous methods are used to avoid phagocytosis of macrophages in vector design. Antibodies are being widely used for tar-

getting non-viral gene delivery. However, the constant fragments can be recognized by phagocytosis and then uptaken by macrophages. Therefore, antibodies that lack constant fragments are sometimes used to help non-viral vectors avoid recognition and clearance by macrophages *in vivo* [83].

Other vectors can also be recognized by macrophages. As discussed above, some cationic polyplexes or lipoplexes will be tagged by some opsonins and then recognized *in vivo*. PEGylation is widely used to avoid the *in vivo* clearance effect by phagocytosis. The highly hydrophilic nature of PEG produces a hydration shell around its conjugated partner, hence reducing intermolecular interactions and, consequently, toxicity [84]. As an effect of reducing intermolecular interactions, PEGylation can effectively avoid phagocytosis; moreover, *in vivo* studies have reported on long circulating half-life of PEGylated vectors [85].

However, some studies have shown that PEGylation can reduce the efficiency of vectors [84] possibly because PEGylation may inhibit cellular uptake and endosomal escape of the vectors. One study compared non-PEGylated and PEGylated liposomes, with the data showing that PEGylated liposomes have poor endosomal escape capability as non-PEGylated liposomes can escape from endosome efficiently [86]. The inhibitory effects of PEGylation depend on some factors. A study about PEGylated cationic liposomes demonstrated that acid-labile PEGylation liposomes have higher transfection efficiency than acid-stable PEGylation ones, which can be ascribed to the more efficient endosomal escape activity of acid-labile PEGylation liposomes [87]. The possible mechanism involved here is that the PEG of acid-labile PEGylation liposomes can be cleaved under low pH (endosomal compartments), allowing the vector to fully interact with the endosomal membrane. So other biodegradable shielding methods should be better than classical PEGylation. According to this hypothesis, recently, an alternative to PEGylation was designed. This work reports, for the first time, the use of hydroxyethyl starch (HES) for the controlled shielding/deshielding of polyplexes. Non-viral delivery systems can be protected by HES shielding, and the HES can then be degraded *in vivo*, indicating that HES shielding has less influence on the efficiency of vectors compared with PEGylation [88].

6. Conclusion

In summary, cellular uptake is the most important intracellular process. Understanding cellular uptake mechanisms is essential to determining the limits of gene delivery. Different pathways have different intracellular fates. Some vectors can enter cells via endo-lysosomal pathways. Thus, some methods have to be used to protect genes against degradation in lysosomes. Optimizing CvME can successfully deliver genes by avoiding endo-lysosomes. Each pathway has its own disadvantages, and learning how to inhibit certain pathways is significant in some cases. In conclusion, taking advantage of cellular uptake mechanisms and knowing how to control them hold considerable potential for improving the efficiency of gene delivery.

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Polylipid Nanoparticle, a Novel Lipid-Based Vector for Liver Gene Transfer

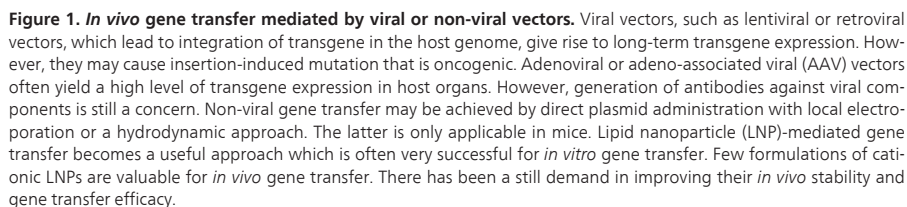
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1. Introduction

Lipid nanoparticles (LNP) are invaluable carriers for drug and gene delivery, and they are classified as cationic, neutral and anionic depending on the electronic charges existing on the surface of the vesicles [1]. These charges are originated from the charged lipids from which lipid nanoparticles are formulated. Cationic LNP are commonly used for DNA or RNA carriers due to their interaction with negatively-charged nucleotide. Both neutral and negatively-charged LNPs are used for drug delivery [2] and may be formulated as sterically stable LNPs (SSLNPs), which are amendable for cell type-specific or tissue-specific targeting delivery [3]. For liver drug delivery, tremendous efforts have been made to develop cell type-selective lipid-based drug carriers. Effective approaches in targeting hepatocytes, Kupffer cells and hepatic stellate cells have been evaluated in small animals [3, 4], and some of them may be translational to clinical application [5]. These approaches are referable when cationic LNPs are considered for cell type-selective gene delivery. A prerequisite for the success of gene therapy for liver disorders is the development of powerful gene carriers. Non-viral vectors have been very successful for gene transfer in an *in vitro* setting, in terms of efficiency of lipofection, applicability in variety of cell types, and amending ability of cell type-specific delivery (Fig. 1). The clinical application of LNP-mediated gene transfer has been hampered by low efficiency, instability in the bloodstream, short-term transgene expression and toxicity. These shortcomings are the bottle neck hindering the gene transfer employing LNPs as carriers for delivery of function gene(s) to solid organs, and are the challenges in moving from small to large animals of potential gene carriers and approaches, and in the translation to clinical application. However, the polylipid nanoparticles (PLNP) we have developed over the past decade represent one of the few formulations that are applicable for *in vivo*



There are a number of critical components for a potential gene therapy product to move from one step to the next in this pipeline. Promisingly, LNP-mediated gene transfection for the treatment of genetic and metabolic disorders or tumors has been moved to clinical trial phases (<http://clinicaltrials.gov>). A phase I pilot study of gene therapy for cystic fibrosis us-

ing cationic liposome-mediated gene transfer (NCT00004471) has been completed. A phase I trial of intratumoral epidermal growth factor receptor (EGFR) antisense DNA delivered by DC-Chol liposomes in advanced head and neck cancer, including oral squamous cell carcinoma (NCT00009841) and DOTAP-Chol-Fus1 liposome-mediated gene therapy for non-small cell lung cancer (NCT00059605) [9] were conducted respectively by University of Pittsburgh and MD Anderson Cancer Center in collaboration with the National Cancer Institute (NCI). Fus1 is a tumor suppressive gene that has been shown to be effective in suppressing the growth of original or metastatic lesions of non-small lung cancer when it is delivered locally or systemically [10]. Thus, it appears that genetic therapy using LNPs as gene carriers has the potential to be specially tailored for genetic disorders or cancers.

2. Nanoparticle carriers for drug or gene delivery

Lipid-based gene carriers include liposomes (cationic or anionic), polymer and dendrimer nanoparticles. Cationic liposomes are capable of delivering genes to cells or tissues, and achieving maximal therapeutic efficiency with minimal adverse effects [1]. However, the use of cationic LNPs for *in vivo* DNA transfection is hindered by substantial problems; i.e. after intravenous administration, cationic LNPs bind to plasma protein and blood cells due to charge reaction. The resulting aggregates of carriers with proteins or cells block microcirculation or may be cleared rapidly [11, 12]. The common formulations for *in vivo* gene delivery are DOTMA or DOTAP-DOPE or DOTAP-cholesterol (Chol). These formulations are highly serum-reactive [6, 13]. Lungs are the major organ shown to be highly transfected probably due to the accumulation of aggregates of lipoplexes with serum proteins or blood cells when the lipoplexes are administrated intravenously [14]. For this reason, cationic LNPs were once used widely for gene delivery to the lungs; and later for treating lung cancers and metastasis with further optimization [10, 15, 16]. LNP-mediated gene delivery to the liver is more difficult than to lungs. For the development of the gene carriers, cationic LNP formulations, such as DC-Chol, DOTAP-Chol, are available for delivering genes to various tissues [17]. A few LNP formulations targeting hepatocellular carcinoma (HCC) have been developed for improving efficacies of drug therapy [18, 19]. In order to avoid the rapid clearance by the reticuloendothelial system (RES) and to increase the drug delivery through the enhanced permeability and retention (EPR) effect to a tumor site by passive targeting, novel strategies, such as reducing particle size, minimizing rigidity of lipids, generating amphiphilic vesicles and shielding from the recognition by RES system, have been attempted in formulating lipid-based drug/gene carriers [1, 2]. To reduce lysosomal degradation, pH-sensitive LNPs are prepared for drug or gene delivery [20]. These approaches may be instructive in the development of LNPs for gene transfer at different stages of preclinical translation.

Polymeric non-viral vectors have exhibited additional advantages of lower toxicity and immunogenicity [21, 22]. These vectors may offer the possibility of industrial production following good manufacturing practice (GMP). Amphiphilic polyethylene glycol (PEG) has been engineered as a linker, most for coupling peptides to cationic lipids. Other polymers,

such as dendritic poly(L-lysine)-b-poly(L-lactide)-b-dendritic vector [23], poly (ethyleneimine) (PEI) [24], poly (methacrylate) [25] and polyamidoamine dendrimers [26], have been demonstrated to be effective for *in vitro* gene delivery. However, striking issues still exist for cationic polymers regarding whether they are applicable for *in vivo* gene transfer to solid organs such as the liver, without significant adverse effects.

3. Liver-specific gene delivery

Because of our interest in gene therapy of liver disorders, we have focused our efforts on improving liver-based gene delivery. The pathogenesis of liver injury and fibrosis involves complicated interactions among different cell populations in the liver, soluble factors, such as cytokines and reactive oxygen species (ROS), and the extracellular matrix components. In order to improve the efficacy in preventing hepatocellular injury, the use of LNPs that are capable of delivering hepatoprotective agents to the liver, selectively to hepatocytes, will increase local concentration of therapeutic agents, reduce adverse effects, and achieve maximal therapeutic efficiency. The parenchymal cell type in the liver is hepatocytes, which are responsible for an array of metabolic function in the body and are often damaged in a variety of pathological processes. The asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes provides a unique means for the development of liver-specific drug or gene carriers. The abundant receptors on hepatocytes specifically recognize the natural ligands, lectin and asialofetuin (AF), as well as those with terminal galactose or N-acetylgalactosamine residues, and hepatocytes endocytose these ligands for an intracellular degradation process [27, 28]. The use of its natural or synthetic ligands, such as galactosylated cholesterol, glycolipids or galactosylated polymers to label LNPs has achieved significant targeting efficacy to the liver [4, 28]. AF-labeled LNPs have been used for improving liver-targeting gene transfer in small animals [29], yet there have not been successful reports available in the translation to large animals, such as pigs [30]. Instead, plasmid DNA was directly administrated into the hepatic vein through a catheter with a balloon closure of hepatic vein blood flow [30]. One particular attention has been drawn in terms of the use of AF-labeled drug carriers for HCC targeting. The expression of ASGP-R in HCC cells varies depending on the differentiation status of HCC cells [31]. In general, well-differentiated HCC usually expresses relatively high levels of hepatocyte-specific genes, including ASGP-R; whereas poorly-differentiated HCC expresses minimal or no hepatocyte-specific genes, including ASGP-R [32]. In most cases, there exists the dramatic heterogeneity of liver-specific gene expression in human HCC tissues [33], and decreased expression of ASGP-R was observed in liver cancer tissue [34]. Therefore, using AF or other galactosylated or lactosylated residues to label LNPs for drug or gene delivery may not always be effective for patients with HCC, because HCC develops on a variety of disease backgrounds and there is a striking variation in ASGP-R expression levels in HCC from different patients. Using well-differentiated hepatoma cells, such as HepG2, Hep3B and Huh-7 cells, as an *in vitro* screening tool may not necessarily reflect targeting efficacy to tumor-specific distribution *in vivo* [35].

High density lipoprotein (HDL) has a high drug carry capacity, and can be recognized by HDL receptors on hepatocytes. Recombinant HDL was utilized to deliver an anti-HBV peptide (nosiheptide) to the liver, and it was shown to achieve a selective distribution in hepatoma cells *in vitro* and a preferential liver distribution in rats [36]. Apolipoprotein E is cleared by hepatocytes, and it has been employed to be carriers for small interfering RNA (siRNA) delivery to hepatocytes [37].

Given the fact that hepatic stellate cells (HSCs) are the major cell type responsible for hepatic fibrosis, a repairing process that causes excess production of extracellular matrix components and deposition of fibrotic scarring in chronically injured liver [38], much attention has been focused on targeting this cell type in the last decade. A couple of cell surface molecules that are overexpressed on activated HSCs during hepatic fibrogenesis, such as insulin growth factor receptor II [39], collagen type VI and platelet-derived growth factor (PDGF) receptor β -subunit [40] are selected as the cell surface targets. Drug carriers labeled with specific peptides recognizing these cell surface molecules, such as cyclic peptide containing arginine-glycine-aspartate (RGD)-labeled sterical lipid nanoparticles [3] or Mannose-6-phosphate human serum albumin (M6P/HAS) [41] exhibited HSC-selective distribution. The RGD cyclic peptide was recently used as a targeting molecule for the recognition of activated HSCs in two animal models for early diagnosis of hepatic fibrosis with a SPECT imaging modality [42]. Using the retinol binding protein (RBP) in activated HSCs seems to be very effective in delivering siRNA against gp46 (rat homolog of human heat shock protein 47), and inhibiting fibrosis in two animal models [43].

Targeting approaches for drug or gene delivery to other non-parenchymal cell types, such as Kupffer cells or sinusoidal endothelial cells, are summarized recently [27]. These approaches are crucial in delivering agents which are anti-inflammatory or anti-oxidants to these cell types due to the fact that Kupffer cells are pivotal in the mediation of inflammatory responses and subsequent fibrogenesis [44].

4. Polylipid nanoparticle-mediated liver gene delivery

Compared to drug delivery, LNP-mediated *in vivo* gene delivery is still in its development stage; and many issues that affect delivery approaches and efficacy remain to be solved. The main issues include: 1) the formation of aggregates between cationic lipids and serum proteins bearing negative charges; 2) the administration routes of LNP-DNA complexes (lipoplexes); 3) intracellular trafficking from the cytoplasm to the nucleus; 4) the proliferative state of cells to be transfected; and 5) transient transgene expression for a short duration [6, 45]. Substantial efforts have been made to address these issues in our previous studies and by others [8, 46, 47]. Particularly, we polymerized an acrylamide lipid to generate a polycationic lipid (PCL), which was able to interact with plasmid DNA effectively and form compacted complexes as demonstrated by Raman microspectral analysis [8]. PCL has a unique molecular configuration and molecular weight distribution as indicated by mass spectrophotometrical analysis [8]. Moreover, this lipid can be synthesized in a multiple gram quan-

tity in a laboratory, and the synthetic approach is amendable for industrial production at a quantity sufficient enough for large animal use [8]. PLNP was formulated with a neutral lipid, cholesterol. The PLNP size was reduced to approximately 100 nm in diameter [7], and the Zeta potential of PLNP was decreased to neutral by neutralizing extra-positive charges with excess plasmid DNA [8]. Not only was this formulation of PLNP non-toxic, but it also displayed transfection efficiency equivalent to other commercially available transfection agents, such as Lipofectamine in hepatoma cell lines [7]. Moreover, high-resolution fluorescent deconvolution microscopy documented that PLNP-mediated gene transfection led to earlier GFP expression in hepatoma cells than Lipofectamine [8]. The unique feature of this formulation is that it is extremely serum-resistant, and exposure to cell culture medium containing 50% fetal bovine serum for 24 hours did not affect its size significantly. PLNP reacted up to 30-fold less with serum proteins or blood cells after intravenous administration in comparison with DOTAP-DOPE or DOTAP-Chol formulations [6]. This feature makes PLNP formulation particularly useful for *in vivo* gene transfer. In the subsequent studies, we have proved that it is very effective in the transfer of reporter genes or function genes to normal mouse livers as demonstrated in Fig. 2 by bioluminescent imaging of firefly luciferase gene expression 24 hours after portal vein injection of PLNP-plasmid DNA complexes (polyplexes) or preclinical models [48, 49].

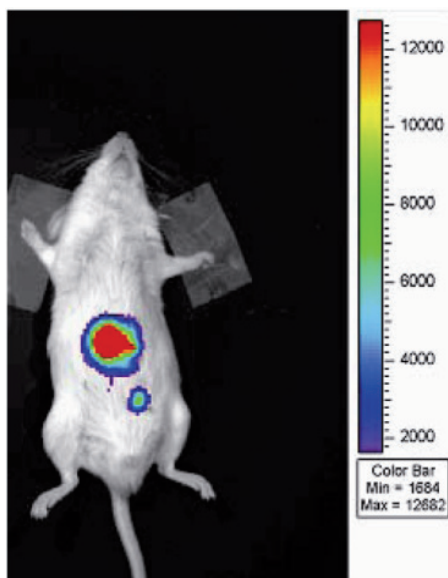


Figure 2. PLNP-mediated gene transfer into mice through portal vein injection. One day after the intravenous injection of polyplexes with pNDLux.2 plasmid encoding the firefly luciferase gene, the animal was imaged by CCD camera. The expression of luciferase was clearly shown in the liver area, demonstrating the effectiveness of this delivery approach and the applicability of a non-invasive imaging modality in the determination of transgene expression in animals.

We also developed an approach to promote normal hepatocytes to proliferate *in situ* without partial hepatectomy, which favors the transgene expression by lipofection but is not acceptable for clinical application [6]. Furthermore, placing an indwelling catheter in the portal vein allows repeated administration of polyplexes for sustained transgene expression [6]. All these efforts render our formulation of PLNP distinct from other lipid-based nanoparticles. Our animal experiments have clearly demonstrated that PLNP is characterized as extremely stable in the bloodstream, and highly effective in liver-based gene transfer when polyplexes are administrated through the portal vein [6, 17]. In comparison with other commonly used lipid formulations of nanoparticles, our formulation possesses the notable advantages essential for *in vivo* gene delivery as illustrated in Table 1.

Characteristics	PCL	PLNP	Lipofectamine	DOTAP-Chol
Cationic lipid	Yes	LNP	LNP	LNP
Particle size (nm)	Irrelevant	125±54	358 ±85	110±20
Size changes(50%FBS)	Irrelevant	100±20nm	2206 ± 311 nm	1050±100 nm
<i>In vitro</i> transfection efficiency Luciferase activity (in RLU)	Irrelevant	>10E7	>10E7	>10E7
Cytotoxicity (LDH release)	Low or none	Normal	10±3% (>5%)	11±3.5% (>5%)
Binding rate to serum protein	Low	Low	Obvious	20-30-fold higher than PLNP
<i>In vivo</i> stability	Irrelevant	Stable	Not determined	Instability
Usage	Raw material for PLNP	<i>In vitro</i> or <i>in vivo</i> transfection	<i>In vitro</i> transfection	<i>In vivo</i> transfection

The content in this table was summarized according to our previous publications [6-8]. FBS = fetal bovine serum. RLU = relative light unit. LDH = lactate dehydrogenase.

Table 1. Comparison of common transfection agents for *in vitro* and *in vivo* application

5. Preclinical trials for proof of the concept

In order to demonstrate that our PLNP formulation is effective in delivering functional genes to the liver, we established a liver injury model in mice caused by the treatment with D-galactosamine (D-Gal) and lipopolysaccharide (LPS). This combination of D-Gal/LPS treatment resulted in a profound acute liver injury characterized by massive liver cell death through apoptosis, elevation of serum alanine aminotransferase (ALT), significant oxidant stress, depletion of the reduced form of glutathione and enhanced lipid peroxidation [50]. In

separate studies we have demonstrated that anti-oxidant enzyme such as extracellular superoxide dismutase (EC-SOD), SOD mimetics (MnTBAP) and catalase are effective in the prevention of hepatic toxicity caused by xenobiotics in primary hepatocytes or hepatoma cells [51-53], and they improved recipient survival and graft function and growth after small-for-size liver transplantation in rats [54]. Therefore, we chose the human EC-SOD gene as a functional gene to prove the feasibility. The EC-SOD gene product was exclusively secreted into the extracellular space and functions as an ROS scavenger. ROS are generated in both intracellular and extracellular spaces, and superoxide anions and hydrogen peroxide (H_2O_2) are able to cross the plasmatic membrane to enter the extracellular space [17]. It was found that two days after portal vein injection of EC-SOD polyplexes, liver EC-SOD gene expression was increased approximately 50-fold compared to the group receiving injection of control plasmid polyplexes, and serum SOD activity was increased accordingly. On the other hand, serum ALT was reduced to nearly one third in mice receiving EC-SOD polyplex injection compared to those with D-Gal/LPS challenge, along with improved liver histology, restored glutathione levels and decreased lipid peroxidation [48]. The findings of this pre-clinical trial confirmed the effectiveness of PLNP-mediated EC-SOD gene delivery to the liver, and that the delivery protected the mice from oxidant stress-associated liver injury. The results also indicate that this anti-oxidant gene delivery approach could be useful in attenuating xenobiotics or drug metabolite-induced toxicity to the liver.

Ischemia/reperfusion (I/R)-associated donor organ damage is inevitable in all solid organ transplantation, and is caused by enhanced oxidant stress with release of inflammatory cytokines, such as tumor growth factor- α (TNF- α) and interleukin 2 (IL-2). Although the precise molecular mechanism of the I/R-associated liver injury remains to be investigated, enhanced oxidant stress with release of superoxide anions or H_2O_2 , depletion of the reduced form of glutathione and increased lipid peroxidation has been the key element in the pathogenesis in orthotopic liver transplantation (OLT) or small size liver graft transplantation (SSLGT) [54-56]. Thus, it is rational to use of antioxidant gene transfer to minimize oxidant stress and improve the donor organ quality and function after the implantation. We delivered either EC-SOD, catalase gene or in combination, using the same approach as described above. Two days after the delivery, the transgene expression was increased for 10-50-fold, with increased SOD or catalase activity in the mouse liver. This delivery led to a marked decrease in superoxide anion levels and H_2O_2 release along with a decrease in serum ALT levels, liver lipid peroxidation and dramatic improvement of liver histology [49]. This study was positively commented by two well-known hepatologists from Europe as an editorial, quoting "beyond a proof of the principle, the study could be the basis for studies with larger animals and may help bridge the gap between the basic understanding of pathophysiologic processes in animal models towards a practical clinical application in liver transplantation" [57]. The findings are especially applicable in living donor liver transplantation, for which small or margin donor livers were used for transplantation. Much more pronounced oxidant stress, a higher rate of graft failure, and retarded graft growth are found in small size liver transplantation than OLT [54, 58]. The margin grafts with small size or steatosis and fibrotic deposition are often used for transplantation in clinics due to severe shortage of donor organs.

6. Challenges in scaling-up and moving towards clinical applications

Our preclinical studies were performed in mice, and there are certainly a number of issues to face when this anti-oxidant gene therapy approach is considered to be evaluated in middle or large size animals such as rabbits, dogs, monkeys or pigs. The first issue is to scale-up, which includes the plasmid DNA generation, synthesis of PCL in a quantity, and formulation of PLNP at a volume sufficient enough for the use in large animals. More challenges exist regarding how to stimulate liver cells to proliferate in large animals and deliver polyplexes locally to the liver. Using a catheter through the femoral vein or jugular vein for retrograde administration into hepatic vein or passing into the portal vein for administration similar to the transjugular intrahepatic portosystemic shunt (TIPS) procedure, which is used to lower portal hypertension in cirrhotic patients, should be feasible in large animals when angiography and the administration are performed by an experienced specialist with the availability of angiographic devices. The latter method was used to administer adenoviral vector in baboons [59]. One trial of plasmid DNA injection into the hepatic vein by blocking the hepatic vein out-flow with an inflated balloon achieved high gene expression levels in selected pig liver lobes [30]. Safety concerns include amount of polyplexes to be administered locally and the effects of the plasmid DNA, PLNP and polyplexes on the liver as well as systematically. LNP-mediated gene transfer is usually transient; therefore, there will be less concern for long-term effects of the transgene products on the host. However, immune reaction to human gene products in animals may occur if the transgene products are produced at sustained levels for a long period of time. It is preventable by administration of immunosuppressive agents, such as FK506. Moreover, innate immunity to plasmid DNA with bacterial unmethylated CG dinucleotide (CpG) can be eliminated by using CpG-free plasmid [60].

An additional concern is to establish a liver injury model to evaluate the effect of anti-oxidant gene transfer by PLNP in large animals. For pigs, exposure to a loading dose of 0.25 g/kg, maintaining the blood concentration of acetaminophen at 350-450 mg/dl, and adapting enteric maintenance dose of 1,000-3,000 mg/hour resulted in the onset of acute liver failure (prothrombin time value <30%) within 32±4.4 hours, and further mortality in 15.8±2.4 hours [61]. A large dose of acetaminophen intake causes significant oxidant stress and acute liver injury due to its metabolism and generation of an interactive metabolite, n-acetyl-p-benzoquinone imine (NAPQI), which binds to the cytoplasmic membrane, leads to lipid peroxidation, depletion of antioxidants, such as glutathione, and results in hepatic injury. Not only will the delivery of antioxidant genes with PLNP in a pig model of liver injury assess the therapeutic efficacy, but also take advantage of a regenerative response to the injury for high transgene expression. Alternatively, small size graft liver transplantation (SSGLT) at ≤50% graft volume could be performed in rabbits or pigs to mimic living donor liver transplantation in humans. Significant oxidant stress-associated injury and regenerative response in the small size grafts will be the best fit for the high transgene expression and ROS scavenging property of the gene product. Therefore, SSGLT may be considered to be a valuable model for evaluating the feasibility and efficacy of anti-oxidant gene transfer for small-for-size-associated graft failure in a transplant setting.

In summary, moving promising PLNP-mediated antioxidant gene transfer from small animals to large animals may face more challenges than discussed above, and it is even more challenging when further considering for clinical use, in terms of safety concern and administrative approval. Fig. 3 provides a schematic illustration of the roadmap from bench to bedside of a potential biological therapy. The reality is that with limited funding opportunities from governmental or private agencies, to cope with multi-facet challenges at a large scale, it is less likely to reach the final goal in a short term. Attracting financial investments and taking advantages of cutting-edging technologies and vast resources from biopharmaceutical companies may advance this process in a fast pace. In this context, the net benefits would be the early clinical application of this promising antioxidant gene transfer in patients with critical needs and the financial return from the investment. We would foresee such a movement occurring in the near future.

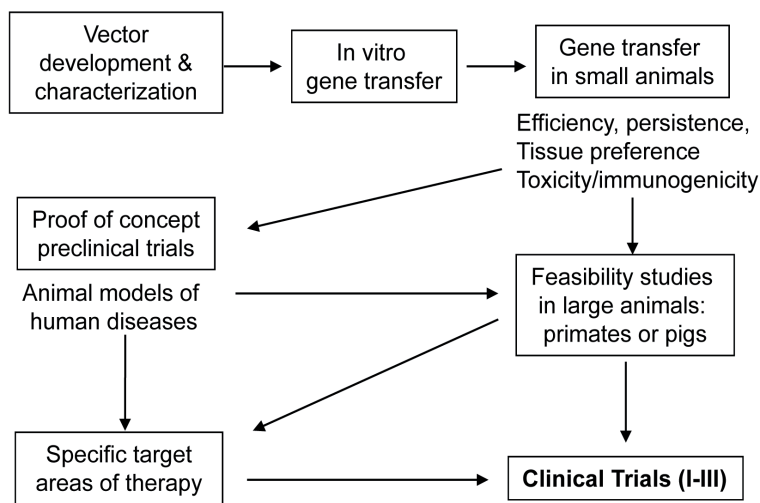


Figure 3. Translation roadmap of a potential gene therapy platform from bench to bedside. This illustration summarizes the major steps in moving a potential gene transfer approach from laboratory research to clinical trials. The actual actions could be more complicated than described. However, for the guarantee of patient safety, each new therapeutic agent must be well characterized, and evaluated in preclinical settings, and then move to large animals for feasibility assessment. The balance between therapeutic benefits and potential risks of an innovative therapy platform always leans on the patient safety as the first priority.

7. Conclusion and prospectives

Non-viral vector-mediated gene transfer has less concern in terms of integration-associated long-term transgene expression and insertion-induced mutation. In general, non-viral vector elicits minimal immune responses in contrast to adenoviral vectors [17]. However, non-viral

vectors, such as lipid nanoparticles (LNPs) possess their own drawbacks when they are considered for *in vivo* use. One prominent issue is the interaction of cationic LNPs with serum protein and blood cells, and this causes a series of issues, such as instability of the lipoplexes or polyplexes and adverse effects to the host, including non-preferential distribution, embolism of the aggregates of lipoplex-protein or blood cells, and inflammatory responses. For these reasons, many gene transfer agents are very effective in cell culture; whereas they have less applicability *in vivo*. Up to date, only a few formulations of cationic LNPs have proved to be effective and safe in animals and have reached the stage of clinical trials, such as DO-TAP-Chol and DC-Chol. Our PLNP formulation has a superior stability profile, and displayed much less reactivity to serum proteins and blood cells when compared to other commercially available formulations. At the same time, it has proved to be the most effective liver-based gene transfer agent [6]. Two preclinical trials with different models of oxidant-stress-associated liver injury have demonstrated the effectiveness of the anti-oxidant gene delivery in the liver, and the efficacy of the gene delivery in minimizing oxidant-stress, attenuating liver cell death, and improving liver histology [48, 49]. Further efforts have been made to move this promising PLNP-mediated anti-oxidant gene transfer technology from bench to bedside. The strategies in pushing this movement towards clinical trials include: 1) Scaling-up of the polycationic lipid production and generation of PLNPs; 2) Generation of specific antioxidant gene plasmids in a GMP facility at the standard for clinical use; 3) Establishing large animal models for safety and efficacy assessment; and 4) Preparation for obtaining administrative approval of clinical application. Although the clinical translation of this potential technology will need tremendous efforts, we anticipate that this technology will eventually reach to patients with critical needs as a novel therapy. Potential indications which may benefit from this therapy range from alcohol or drug toxicity to living donor liver transplantation with a margin graft. This technology is also applicable in oxidant stress-associated disorders in other systems, such as ischemic cardiac, pulmonary, brain or renal damage, etc. [17]. With the combination of our extensive expertise in drug and gene delivery, advanced knowledge and skills in liver injury, fibrosis, transplant and cancer research and practice, in addition to the engine of financial investment from various sources, such as venture capital and governmental support in entrepreneurship, we are optimistic to foresee the benefits of this technology in indicated patients in a near future. Nevertheless, the road to reach this goal will not be smooth, and various challenges demand powerful solutions.

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Abbreviations used in the chapter

ASGP-R = asialoglycoprotein receptor; DOTAP = (dioleoyloxy)-3-(trimethylammonio) propane; DOPE = L- α dioleoyl phosphatidylethanolamine; EC-SOD = extracellular superoxide dismutase; HCC = hepatocellular carcinoma; LDLT = Living donor liver transplantation; LNP = lipid nanoparticles; OLT = orthotopic liver transplantation; PEG = polyethylene glycol; PLNP = polylipid nanoparticles; polyplex = PLNP-plasmid DNA complex; RES = reticuloendothelial system.

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DNA Electrotransfer: An Effective Tool for Gene Therapy

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Additional information is available at the end of the chapter

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1. Introduction

The concept of gene therapy was first introduced in the mid-80s, and is based on the delivery of genetic material (DNA or RNA) in the nucleus of patient cells, so that it is expressed and produces a therapeutic effect.

Different approaches can be considered:

- Correcting defective function by supplying a functional gene to the cells, thereby directly addressing the cause of a genetic disease.
- Transferring a gene encoding a therapeutic protein, in order to treat, prevent or slow the progression of certain diseases.
- Introducing a gene leading to the death of a diseased cell
- Introducing antisense DNA inhibiting the formation of a protein or the replication of a virus

Originally developed for monogenic diseases, and therefore associated with the compensation of genes whose alteration is responsible for diseases, the concept of gene therapy has rapidly expanded to the use of DNA as a new type of drug. Therefore, gene therapy leads to indications which are far beyond the case of genetic diseases, since a DNA drug can, in principle, replace any medication which will control protein synthesis. Gene therapy seems an alternative choice to fight against diseases currently treated imperfectly, or not treated with conventional pharmaceutical approaches.

In addition, gene therapy has many advantages compared to the administration of recombinant proteins. Indeed, recombinant proteins are costly and their elimination from the blood

flow is fast, while gene therapy leads to a long-term and potentially regulated production of a therapeutic protein. Gene therapy also allows the localized expression of the transgene, avoiding any risk associated with the presence of a systemic exogenous protein.

The main limitation of current gene therapy is the development of effective gene transfer. Indeed, in order to reach the cell nucleus, the therapeutic gene has to cross several biological barriers. Therefore, the success of any gene therapy requires the development of efficient and appropriate methods and vectors for introducing the gene of interest into target cells. The ideal vehicle for gene transfer must have the following properties: (1) specificity to target cells, (2) localized gene delivery, (3) resistance to metabolic degradation and/or attack by the immune system, (4) minimum side effects, and (5) eventually controlled temporal transgene expression [1].

Many methods of *in vivo* gene transfer exist and are generally classified into two main categories: viral and non viral. Viruses are very efficient vehicles for gene transfer; however their use is limited by high production costs and safety concerns, such as immune response, possible pathogen reversion, mutagenesis and carcinogenesis. Considering these limitations, the delivery of therapeutic genes to target cells by non viral approaches may be of great value for the development of gene therapy. Among these approaches, *in vivo* electroporation, also called *in vivo* electroporomeabilization or *in vivo* electrotransfer, has proven to be one of the simplest and most efficient methods for gene therapy, while at the same time being safe, cheap, and easy to perform.

In vivo electrotransfer is a recent physical technique for gene delivery in various tissues and organs, which relies on the combination of plasmid injection and delivery of short and defined electric pulses. This process results in the association between cell permeabilization and DNA electrophoresis. Skeletal muscle have now been frequently electrotransferred, since it offers promising treatment for muscle disorders, but also a way for systemic secretion of therapeutic proteins, by converting skeletal muscles into an endocrine organ: the protein produced can diffuse into the vascular system and circulate throughout the body to exert a physiological and potentially therapeutic effect. Many published studies have demonstrated that plasmid electrotransfer can lead to long-lasting therapeutic effects in various pathologies such as cancer, rheumatoid arthritis, muscle and blood disorders, cardiac diseases, etc... Indeed, the physical method of electrotransfer allows for greater efficiency of gene transfer after a single injection and improves protein expression by several orders of magnitude, as compared to DNA injected in the absence of electrotransfer. Therefore, plasmid electrotransfer can be considered a powerful tool for gene therapy.

The scope of this chapter encompasses the methods of electrotransfer, its implementation, mechanism, optimization and therapeutic applications.

2. Description of the electrotransfer technique

In 1982, E. Neumann and his collaborators demonstrated *in vitro* the possibility of introducing DNA into cells using electrical pulses [2]. These electric pulses would cause the destabi-

lization and permeabilization of the plasma membrane of suspended cells, thus promoting the entry of exogenous DNA into these cells. Two years later [3], the confirmation of this result opened the way for the development of electroporation (or electropermeabilization) into bacterial [4], fungal [5] vegetal or animal cells. This method is routinely used now. The optimization of electrical parameters is critical to allow transient permeabilization, together with a satisfactory cell survival rate [6].

In initial studies, *in vivo* DNA electrotransfer has been tested in the skin in 1991, by the use of exponentially decaying electrical pulses, and in 1996 in the liver using trains of short 100 μ s pulses [7]. In 1998, four independent teams showed the effectiveness of electrotransfer using pulses of long duration (5-50ms): in skeletal muscle, our team in collaboration with that of Luis Mir [8] and Aihara [9], in tumors, Rols *et al.* [10] and in liver Suzuki *et al.* [11]. *In vivo* DNA electrotransfer has now been successfully used in a broad range of target tissues and organs including for example : arteries [12], skin [13], tendon [14], bladder [15], cornea [16], the retinal cells [17], spinal cord [18] and brain [19].

Electropermeabilization can also be used to deliver chemical drugs into the cells: e.g. electrochemotherapy in tumors, with the use of bleomycin, developed since 1991 [20]. Several clinical trials are underway [21], primarily for the treatment of subcutaneous or skin tumors [22, 23] and recently for the treatment of breast cancer with cisplatin [24] (For a review see [25]).

3. Mechanism of electrotransfer at the cell level

The technique of electroporation for the transfer of nucleic acids has been used since the 80s, however its exact mechanism is not yet completely elucidated [26, 27]. At the cell level, it seems that two phenomena occur: first the permeabilization of the cell to small molecules, probably due to a destabilization of the cell membrane, and secondly the transport of DNA by electrophoresis.

3.1. Permeabilization

The lipid bi-layer of the plasma membrane separates two solutions with very high ionic conductivity: the cytoplasm and the extracellular medium. Typically, at rest, the membrane potential difference (ΔV_{m_0}) is around -70mV. When an electric field is applied to a cell, the resulting current induces an accumulation of electric charges at the cell membrane which leads to a variation of this transmembrane potential. And if the transmembrane potential exceeds a certain threshold value, the cell membrane is disorganized and structural changes occur. That is a necessary condition for an effective gene transfer [28].

Shall the cell be considered a hollow sphere where the thickness of the membrane is negligible vis-à-vis the cell radius, then the transmembrane potential difference ΔV_m induced by an electric field is, as described by Schwann's equation:

$$\Delta V_m = f \cdot g \cdot r \cdot E \cdot \cos \theta \cdot (1 - \exp(-t/\tau)) \quad (1)$$

Thus, the transmembrane potential difference ΔV_m is proportional to

- the cell radius (r)
- the magnitude of the electric field (E) (expressed in volts/cm)
- the cosine of (θ), its incidence angle,
- a cell shape factor (f)
- the conductivity of the medium (g)
- the pulse duration for which the electric field is applied (t)
- the charging time constant of the cell (τ).

If the membrane is seen as a pure dielectric object, g is equal to 1. Under the conditions used for cellular electroporation, the pulse duration is significantly longer (of a few hundred microseconds to a few milliseconds) than the charging time constant of the cell, which is of the order of a few microseconds. The equation can be simplified to:

$$\Delta V_m = f \cdot r \cdot E \cdot \cos \theta \quad (2)$$

This transmembrane potential difference ΔV_m is not uniform on the surface of the cell: the induced transmembrane potential is maximal at the points of the cell facing the electrodes ($\theta = 0$ and π).

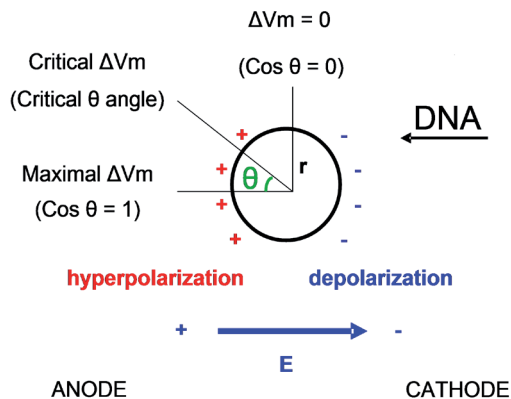


Figure 1. Theoretical model of the cell for electroporation: E , the electrical potential induces ΔV_m , a transmembrane potential difference which dependent on r , the radius of the cell and θ , the angle between the direction of electric field and the normal to the tangent of the membrane of the cell at this point

The membrane is off-balance and becomes transiently permeable when the sum: ΔVm_0 (at rest) + ΔVm (induced) reaches a threshold value of about 200mV [29]. Thus, the greater the difference between the threshold value and the value applied, the greater the surface area is permeabilized. However for a given electric field, beyond a certain angle, the ΔVm falls below the threshold value of permeabilization. The relationship between the applied electric field and the permeabilized surface was demonstrated by *in vitro* fluorescent labeling of permeabilized areas of the cell [30]. Moreover, these studies have shown that it is the face of the cell toward the anode side which is permeabilized first, the negative potential of the cell being in addition to that induced by the external electric field.

One theory suggests that the DNA enters into the cell through pores which are generated by electrical stimulation [32]. The electroporemeabilization creates relatively stable "electropores" [2, 33]. But these pores have never been visualized. The plasmid DNA may optionally pass the membrane after a step of binding to the surface of the cell and by diffusion.

The second phenomenon necessary for gene transfer by electroporation is the electrophoresis of negatively charged DNA.

3.2. DNA electrophoresis

The occurrence of an electrophoretic process has been demonstrated *in vitro* [31]. Various studies have shown this electrophoretic effect: Klenchin *et al.* demonstrated that DNA has to be present at the time of the pulses [31]. Furthermore, they showed that the transfection efficiency depends on the polarity of the electric field. Sukharev *et al.* also showed *in vitro* that short pulses of high voltage (HV) induce membrane permeabilization but not transfection, whereas long pulses at low voltage (LV) do not induce permeabilization or transfection. However, the sequence "high voltage pulses followed by low voltage pulses" provides a transfection. An hypothesis is proposed that transfection of cells permeabilized by high voltage is only possible if low voltage pulses can subsequently mediate DNA electrophoresis [34].

The role of permeabilization and electrophoresis was demonstrated directly at the cell level by fluorescence microscopy [35]. This work shows that interaction between the membrane and electroporemeabilized DNA is induced in response to electrical pulses of a few milliseconds. DNA electrophoretically accumulates on the cathode side of the cell without immediately moving into the cytosol (Figure 1). Thus DNA must be present during the pulse and electrophoresis induced by the electric field promotes its transfer through the membrane, but it is only during the following minute that DNA crosses the electroporemeabilized membrane [36]. There is a direct relationship between the DNA/membrane interaction and transfection efficiency: the larger the contact surface between DNA and the membrane, the higher is the expression [27].

4. Mechanism of *in vivo* electrotransfer

In the early 90's, the first studies about *in vivo* electroporation appeared. They primarily concerned the transfer of chemical molecules. The first real demonstration of *in vivo* cellular

electropermeabilization was performed on tumors after injection of bleomycin, a cytotoxic anticancer agent, [22, 37]. The effectiveness of bleomycin depends on its intracellular concentration, but this drug penetrates poorly into cells. Therefore, a better penetration of bleomycin was measured after application of electric pulses to tumors, leading to an enhanced desired cytotoxicity.

Most studies are pointing to a mechanism of *in vivo* electrotransfer comparable to the mechanism of *in vitro* electrotransfer described above, which can be extended to the whole tissue: several steps have to take place, including cell permeabilization beyond a threshold value of local electric field. In 1999, we evaluated on one hand cell permeabilization following the application of electrical pulses by measuring the ability of muscle cells to capture a small radioactive hydrophilic molecule complex of EDTA Chelating 51 chromium ($^{51}\text{Cr-EDTA}$), and on the other hand, transgene expression for evidence of DNA entry [38, 39]. The uptake of $^{51}\text{Cr-EDTA}$ was similar whether injected thirty seconds before or after applying electrical pulses. In contrast, DNA injected after the electrical impulses does not penetrate into cells. This suggests that DNA must be present *in situ* at the time of electrical pulses to obtain an efficient cell transfection, and that there is a direct, active effect of the electric field on the DNA molecules to promote their entry into cells. Hence the current mechanistic hypothesis of gene electrotransfer necessitates not only a permeabilization of cell membranes but also a DNA electrophoresis.

This hypothesis is supported by the study of Bureau *et al.* [40] of gene electrotransfer in skeletal muscle of mice with different combinations of long pulses of low voltage (LV, i.e. electrophoretic pulses) and short pulses of high voltage (HV, i.e. permeabilizing pulses). Only the combination of a HV-pulse followed by a LV-pulse provided efficient gene transfer. Further studies confirmed that HV-pulses are related to permeabilization, while LV-pulses are related to the efficiency of DNA electrophoresis [41]. The importance of cell permeabilization was also studied by magnetic resonance imaging using a gadolinium complex as contrast agent (dimeglumine gadopentate): the zone of di meglumine gadopentate complex permeabilization is identical to the area expression of electrotransferred DNA [42].

The destabilization of cell membranes and the electrophoretic effect are probably not the only mechanisms involved in gene transfer by electroporation. Scientists have discussed the importance of energy metabolism (ATP and ADP) for the passage of DNA through the permeabilized membrane and its migration to the nucleus [28].

Other studies suggest a mechanism of DNA transport by endocytosis [43]. These same studies show that transfection efficiency does not decrease if the electrical pulses are delivered up to four hours after injection of DNA, while other studies show that most of the injected DNA is degraded in first hours after injection [44]. We also confirmed that after an intramuscular injection, most of the DNA is degraded and eliminated quickly. However, a small proportion of DNA is preserved and provides a source of stable DNA which can be electrotransferred [45].

In summary, the molecular mechanism of *in vivo* DNA electrotransfer is still under investigation. It likely corresponds to multiple steps whose elucidation and understanding of re-

spective contribution could help to develop more effective electrotransfer strategies and protocols.

5. Electrotransfer into practice

The *in vivo* electrotransfer technique is particularly easy to implement: a solution of plasmid DNA (i. e. a circular nucleic acid) in isotonic saline (NaCl, 150mM) is injected into the target tissue with a syringe, and electric pulses are then delivered by means of electrodes placed on either side of the injection site and connected to a generator (Figure 2). Electrodes can be either needles or plates.

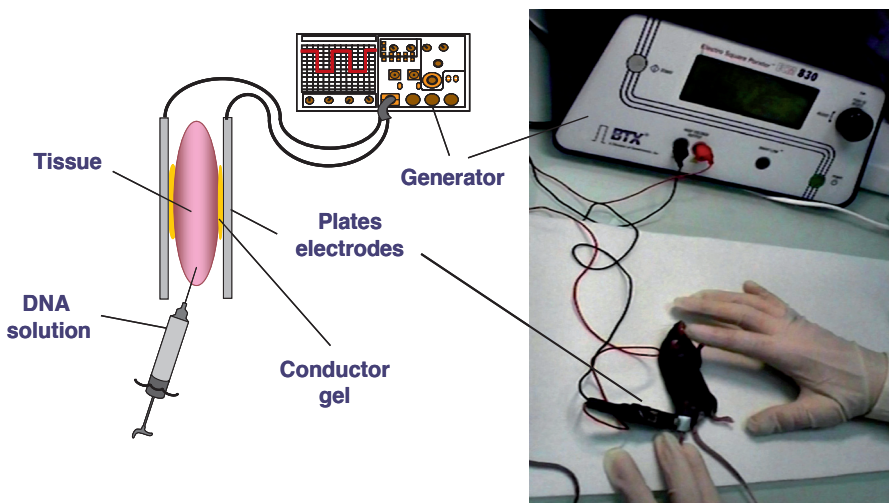


Figure 2. Experimental set up for intramuscular plasmid electrotransfer in mice

This technique allows a site specific gene transfer. It is relatively efficient in skeletal muscle and is applicable to many other tissues such as brain, liver, skin, bladder, kidney, lung, cornea, retina, testis, tumor tissue etc... for more details see [46]. Electrotransfer can also be used in a wealth of animal models, ranging from rats and mice to sheeps [47] and cows [48] and even fish [49].

5.1. Operating parameters

The efficiency of gene transfer depends on the target tissue, the delivered DNA and electric pulses parameters. The aim is to deliver, into each tissue, electrical pulses that can cause the permeabilization of cell membranes and DNA transfer, while remaining below the toxic threshold. Otherwise, local cell death by necrosis of the treated cells would occur, followed

by tissue regeneration, which would induce the loss of the benefit of the treatment (but with no toxicity at the level of the whole organism). Therefore, optimal conditions for the DNA electrotransfer in a targeted tissue result from a compromise between the efficiency of DNA transfer and minimal cellular toxicity.

5.1.1. The electrodes

The choice of electrodes depends on the target tissue and the size of the treated animal. It is critically important and should be carefully considered. For an electrotransfer on a small animal in a tissue such as skeletal muscle, or liver tumor, most experimenters use electrodes made of two plates attached to a clamp (Figure 3, left). Indeed, this type of electrodes can be easily applied externally on each side of the interested tissue. Because one key parameter is the electric field, which is related to the ratio between the voltage applied and the distance between electrodes, this latter distance should not be too large in order to avoid prohibitive high voltage *in vivo* delivery. Thus, for animals of larger size, needle electrodes (Figure 3, right) are more often used than external plates.

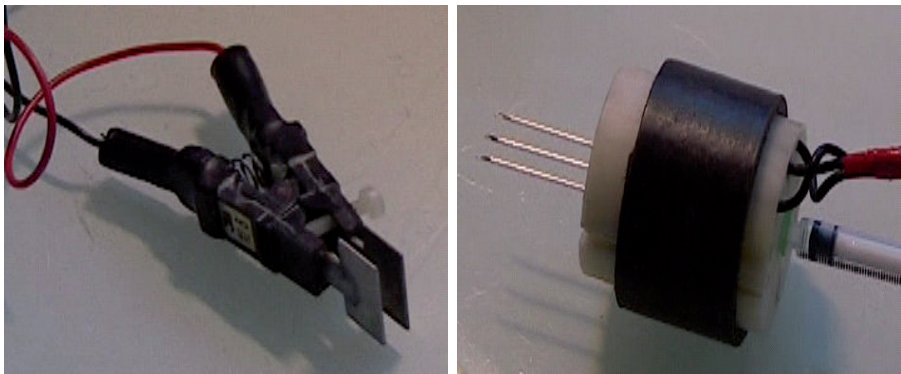


Figure 3. Examples of electrode plates for external use (left) and needle electrodes for internal use, designed by the company Sphergen (right).

5.1.2. Electrical parameters

Knowing the magnitude and distribution of electric field is very important for both efficient gene transfer and reduced toxicity. The distribution of the electric field is dependent on both the tissue and the type of electrodes, which causes variations in the effective magnitude of the field in the tissue area of interest. The electric field distribution is more homogeneous when using plate electrodes than with needle electrodes, and for a given setting, the resulting electric field is lower with needle electrodes than with electrode plates [38].

Moreover, it is necessary to determine, for each tissue and each species, the threshold values of the electric field magnitude, i.e. the permeabilization threshold (reversible) and the cell

damage threshold (irreversible), in order to define optimal electrical conditions for gene transfer with minimal toxic effects. Micklavic *et al.* have developed a system combining numerical predictions and experimental observations in order to determine these thresholds in the case of needle electrodes used in rat liver for drug delivery [50].

Different types of electrical pulses can be applied: unipolar square pulses, bipolar square pulses, or pulses with exponential decay [51]. The exponentially decaying pulses, colloquially referred to as “exponential pulses” are mainly used *in vitro* with a time constant dependent on the resistance of the incubation media. The square pulses are preferred *in vivo*, since the voltage and pulse duration can be set independently of the electrical resistance of the targeted tissue. Unipolar square pulses are the most widely used for electrotransfer experiments, while bipolar squares pulses are rather used for electrophysiology [52].

5.2. Toxicity

Tissue damage can be caused by electrotransfer and thus limits the efficiency of transfection [53]. The cell permeabilization is the main toxicity factor: it leads to an inward diffusion of the external medium as well as leakage of intracellular content, thus changing the composition of the latter. This toxicity can be reduced by minimizing the duration and the extent of permeabilization.

Other factors of toxicity have been described such as oxidative stress due to the formation of free radicals near the electroporabilized membrane [6, 54]. It was also shown that electrotransfer induced muscle damage dependent on the amount of DNA injected [55]; these lesions disappear within two months after injection.

In our laboratory, histological analyzes of muscle slices have shown that the application of electric fields optimized for gene transfer does not induce gene expression markers of stress and cellular toxicity [56]. Other experiments have allowed to conclude that, even in optimized conditions, very little muscle damage is generated: few inflammatory lesions are observed with a maximum in the first seven days after the electrotransfer, but these disappear rapidly in less than three weeks [57-58].

It is also possible to reduce the extent of damage by increasing the accessibility of DNA to target cells. Indeed, studies have shown that improving the plasmid distribution leads to an increase in transgene expression. Thus, the value of the electric field used can be reduced. Better distribution can be obtained for example by pre-injection of hyaluronidase [59], an enzyme that degrades hyaluronic acid, which is a major component of the extracellular matrix [60]. This pretreatment allows for the same expression level, using lower voltages while reducing muscle damage [61]. A pre-injection of sucrose may also improve the distribution of DNA, by creating spaces between the muscle fibers [62]. Similarly, a pre-injection of poly-L-glutamate, an anionic polymer, seems to increase the internalization of the plasmid inside the cell and/or to reduce its degradation [63], and therefore increases the expression of exogenous gene.

5.3. Target tissues

During recent years, electrotransfer has been applied in various animal species to many tissues, including skeletal muscle, skin, liver, lungs, kidneys, joints, brain, retina, cornea, etc... [64]. The optimal parameters of a given electrotransfer should be determined based on the cell type and species, since these parameters strongly depend on tissue organization and the size of the transfected cells.

5.3.1. Skeletal muscle

One of the most widely used tissues for electrotransfer is skeletal muscle. The DNA electrotransfer into skeletal muscle was discovered independently by three teams [8, 9, 52]. Indeed, skeletal muscle offers many advantages:

- a large, easy access;
- sets of muscle fibers are parallel to each other: many fibers might have an optimal orientation relative to the electric field, which promotes even transfer across the entire length of the fibers;
- unlike other cells, muscle cells have multiple nuclei flattened against the cell membrane, which facilitates DNA trafficking to the nucleus;
- muscle fibers do not divide, ensuring long-term gene expression, notwithstanding the absence of regeneration due to injury or cytotoxic immune response;
- finally, a major advantage of skeletal muscle lies in its ability to produce and release biologically active proteins into the bloodstream, due to the strong vascularisation.

Combined together, these features can turn muscle into systemic drug delivery system for distant targets [65]. Interestingly, the cotransfection of multiple unlinked genes can be easily performed by electroporation [66]. For examples of electrotransfer in skeletal muscle in various mammalian species see [46].

5.3.2. The skin

The skin is, as muscle, also a widely used tissue for DNA electrotransfer, mostly because:

- this tissue is easily accessible and a large area of tissue can be treated;
- keratinocytes, which are epidermal cells, can synthesize and secrete therapeutic proteins that reach the bloodstream;
- by its natural function of a biological barrier, the skin contains cells that present antigens and is therefore an organ of choice for applications in DNA vaccination;
- the epidermal cells have a short lifespan, which can be useful for treatments requiring a brief period of expression.

However, skin structure [67] does not facilitate gene transfer. In particular, the top layer (stratum corneum or horny layer) is a major barrier [68, 69]. But a high level of expression in

the skin from a single injection could still be observed [70, 71]. Moreover Dujardin *et al.* have shown that square or exponential pulses induce moderate and reversible effects on the skin without inflammation or necrosis, while transiently permeabilizing the skin and thus allowing the passage of molecules [72].

5.4. Optimization of *in vivo* electrotransfer conditions

An important goal for gene transfer applications is the level and duration of gene expression. To determine optimal conditions which maximize efficiency while reducing tissue damage, different protocols have been used to improve the access of plasmids to targeted cells. As already described, improved plasmid distribution in the skeletal muscle leads to an increase in DNA expression. Accordingly, Cemazar *et al.* showed recently enhanced transfection efficiency of gene transfer by pretreatment of tumors with hyaluronidase and/or collagenase, two enzymes which modulate components of the extracellular matrix [73].

A secretion signal can be also added to the transgene sequence : we have recently shown that by modifying the cellular localization of the produced protein by adding a secretory signal, the production and secretion of this protein is enhanced, thus enhancing biological effect [74].

We have also shown that codon optimization of the transgene (i.e. retaining the natural amino acid sequence but using the preferred host animal codons) leads to increase in the expression of the protein of interest [74].

Another method to increase the stability of the protein produced in the blood circulation is to increase its size in order to avoid kidney excretion. Thus, the construction of fusion proteins, for instance by fusing a therapeutic protein with an IgG constant [75], appears a simple way to deliver enhanced levels of secreted proteins without altering their biological activities.

The enhanced protein expression, and so their biological effects, also depends of the injection regimen and the administered plasmid dose [74].

6. Applications of plasmid electrotransfer

DNA electrotransfer is a recent technique of has not yet successfully completed all stages of clinical development, but this is progressing. The first Phase I human clinical trial has been initiated in U.S. by the company Inovio Biomedicals, for the treatment of skin cancer [76]. Since then, the delivery of plasmid DNA encoding therapeutic genes has been tested extensively in preclinical melanoma models [77].

Applications designated as "therapeutic" which are mainly reported in the literature have been demonstrated on animal models of human diseases. The main potential therapeutic areas cover cancer [78], cardiovascular diseases [75], autoimmune diseases [79], monogenic diseases [80], organ-specific disorders [81] and vaccination [82, 83]. Different examples show

the efficiency of plasmid electrotransfer to produce therapeutic proteins in various pathologies [46]: all these experiments showed an improvement in symptoms of the relative disorder.

6.1. Cancer

Cancer accounts for major field of application trials of gene therapy. Different strategies can be broadly grouped into four main categories:

- a. Stimulation of the immune response against a tumor [84],
- b. Use of suicide genes [85-87];
- c. Repair cell cycle defects caused by the loss of tumor suppressor genes or oncogene activation [88],
- d. Inhibition of tumor angiogenesis [89].

These strategies can be combined to obtain synergistic results. For example, a combination of HSV-TK-suicide gene therapy and IL-21 immune gene therapy by electrotransfer improves antitumor responses in mice [90]. Moreover, *in vivo* electrotransfer could be used in combination with other strategies such as chemotherapy, because these two approaches use different mechanisms to kill cancer cells, and thus a synergistic effect may be obtained.

Actually, electroporation of DNA encoding cytokines into tumors is extensively studied: IL-12 [91], IL-18 [92], IFN- α [93] have been shown to reduce tumor growth and increase survival times in different tumor models. Other interesting results are represented by the inhibition of tumor growth in various models with plasmids encoding metalloproteinase-3 inhibitor for the treatment of prostate cancers [94], or encoding endostatin for his therapeutic efficacy in mouse-transplanted tumors [95].

All these experiments show the potential of *in vivo* electrotransfer for cancer treatment. And the strategy used, i.e. the direct intra-tumoral plasmid electrotransfer, is well suited for the local production of therapeutic proteins. However, since the efficacy of gene transfer into tumor cells *in vivo* is generally low, intramuscular electrotransfer can also be efficiently used for distal tumor treatment. Indeed, an important application of the technique of plasmid electrotransfer is the protein secretion by skeletal muscle: the produced protein, such as, for instant, an immunostimulating cytokine, can diffuse into the vascular system and circulate throughout the body to exert a physiological effect, particularly therapeutic. This distal approach may be very powerful for surgically inaccessible tumors, such as head and neck tumors.

Finally, the intramuscular electrotransfer of a plasmid encoding the prostate membrane specific antigen (PMSA) has been tested in a human clinical trial of prostate cancer active immunotherapy [96]. DNA fusion-gene vaccination in patients with prostate cancer induces high-frequency CD8 (+) T-cell responses and increases PSA doubling time [97].

6.2. Monogenic diseases

Monogenic diseases with an identified defective gene have been the first diseases targeted by gene therapy approaches. Among these diseases, Duchenne muscular dystrophy (DMD), which is characterized by the absence of dystrophin, is a good model, since even a small amount of dystrophin would be sufficient to reverse the clinical phenotype of the disease. An approach to eventually restore this protein in patients with DMD is to introduce into their muscles a plasmid encoding dystrophin cDNA. Pichavant *et al.* were the first to demonstrate local restoration of full-length dog dystrophin in dystrophic dog muscle by DNA electrotransfer [98].

6.3. Hematopoietic factor deficiency

Erythropoietin (EPO) is another good candidate for gene therapy applications because a small amount will produce the desired physiological effect of raising the hematocrit. Numerous studies, in particular by our own group, report efficient EPO secretion after plasmid electrotransfer, with a therapeutic effect in anemia and beta thalassemia. The use of intramuscular plasmid electrotransfer for EPO gene delivery in mice increased approximately 10 to 100-fold the expression of this gene, as compared to naked DNA alone [99, 100]. Moreover with this method, the protein in circulation and hematocrit levels were stable for 2 to 6 months after a single injection of minimal amounts (as little as 1 μ g) of a plasmid carrying the mouse EPO cDNA. Several studies also showed that EPO expression could be regulated, for instance by co-administering an EPO encoding plasmid under the control of a tetracycline-inducible promoter and a second plasmid carrying the reverse tetracycline-dependent transactivator protein [100, 101]. All these studies exemplified that plasmid DNA electrotransfer can efficiently produce enough amounts of transgenic EPO in normal mice.

In collaboration with the group of Y. Beuzard, we have demonstrated the relevance of intramuscular electroporation of an EPO-expressing plasmid in a mouse model of human β -thalassemia, a severe genetic disease, leading to a durable and dose-dependent phenotypic correction of this severe genetic disease [102]. In addition, we have also shown that it is possible to produce fusion protein by plasmid DNA electrotransfer [103]: indeed since the bridging of two adjacent EPO receptors triggers a conformational change that initiates signal transduction [104], we have hypothesized that the fusion of two EPO molecules might lead an increase in intrinsic activity of EPO. Thus, we demonstrated that the injection of EPO dimer encoding plasmid by electrotransfer in a skeletal muscle of β -thalassemic mice induces an increase in the biologic specific activity of this EPO dimer in comparison with the activity of monomer [103].

Furthermore the secretion peak of therapeutic protein following DNA administration is potentially deleterious. We reported that muscular electrotransfer of low doses of plasmid can be repeated several times to weeks or even months after the initial injection, and that this strategy leads to efficient, long-lasting and non-toxic treatment of β -thalassemic mouse anemia avoiding the deleterious initial hematocrit peak and maintaining a normal hematocrit with small fluctuation [105].

In addition, Gothelf *et al.* demonstrate that gene electrotransfer to skin of even small amounts of EPO DNA can lead to systemically therapeutic levels of EPO protein [106].

6.4. Cardiovascular diseases

Gene therapy is an attractive strategy for the treatment of cardiovascular disease. However, using current methods, the induction of gene expression at therapeutic levels is often inefficient. Therefore DNA electrotransfer directly into heart may enhance the delivery of therapeutic protein as shown the team of R. Heller : the electroporation method ameliorates the delivery of a plasmid encoding an angiogenic growth factor (vascular endothelial growth factor, VEGF), which is a molecule previously documented to stimulate revascularization in coronary artery disease [107]. Ayuni *et al.* demonstrated that, unlike the usual methods to treat coronary artery diseases, electrotransfer applied directly into the beating heart enhances the delivery of a plasmid injected via the coronary veins after transient occlusion of the coronary sinus [108]. These results show that *in vivo* electroporation mediated gene transfer is feasible and safe, in particular to the heart. Finally, in skin, D. Dean reported that using electroporation in skin enhances delivery of plasmid DNA encoding fibroblast growth factor-2 (FGF-2) to induce neovascularization as a therapy for ischemia in a rat model [109].

6.5. Eye diseases

The eye is an isolated organ difficult to reach via systemic administration. Eye diseases are treated with intra- or periocular injections and these repeated injections bear the risk of adverse effects, mainly infections, and are poorly tolerated by the patients. The use of DNA electrotransfer technique is therefore possible to deliver a local treatment. Our team associated with an ophthalmology group has developed electrotransfer to the ciliary muscle, which is a particular smooth muscle with some characteristics of striated skeletal muscle, for the local treatment of inflammatory eye disease. This approach led to production and secretion of therapeutic levels of TNF α soluble receptor in the ocular media, and not in the serum, thus preventing clinical and histological signs in a rat uveitis model [110, 111]. Recently, suprachoroidal electrotransfer with a reporter plasmid to transfect the choroid and the retina without detaching the retina has been reported [112]. Not only choroidal cells but also RPE, and potentially photoreceptors, were efficiently transduced for at least a month, without ocular complications. This minimally invasive non-viral gene therapy method may open new prospects for human retinal therapies.

6.6. Obesity and diabetes

As mentioned above, skeletal muscle can be an efficient platform for the secretion of erythropoietin (EPO), which displays a variety of metabolic effects when it is expressed in supra-physiological levels. Hojman *et al.* have proposed to overexpress EPO in muscle by electrotransfer of plasmid in the aim to protect mice against diet-induced obesity and normalize glucose sensitivity, associated with a shift to increased fat metabolism in the muscles [113]. Similar results were obtained after DNA electrotransfer of plasmid encoding the carni-

tine palmitoyltransferase 1 (CPT1), the enzyme that controls the entry of long-chain fatty acyl CoA into mitochondria: an overexpression of CPT1 led to enhance rates of fatty acid beta-oxidation and improved insulin action in muscle in high-fat diet insulin-resistant rats [114]. In the same model, electrotransfer of the orphan nuclear receptor (Nur77) significantly ameliorates the effect of this protein on glucose metabolism [115].

6.7. Vaccination and passive immunization by antibody production

The prospect of inducing an immune response to a protein expressed *in vivo* directly from administered DNA vaccine represents an attractive alternative to other modes of vaccination. Plasmid electrotransfer has been used in genetic immunization to produce antigenic proteins. It is now well established that genetic immunization induces both durable cellular and humoral responses [116]. This type of immunization is often developed for vaccination (virus or antibacterial), for anticancer active immunotherapy, and also to induce in animals the production with high yield of antibodies against a given antigen.

Since electrotransfer efficiently transfers genes compared to a single injection of plasmid, improving antigenic protein expression by several orders of magnitude, the antibody titer and the quality of the immune response are also improved [117], with an increasing factor of 100 in mice after electrotransfer of a plasmid encoding a surface antigen of hepatitis B [118]. High titers of antibodies were also obtained in mice and rabbits after i.m. electrotransfer of a plasmid encoding an envelope glycoprotein of hepatitis C [119], and in mice after electrotransfer of a plasmid encoding a protein of *Mycobacterium tuberculosis* [120]. In the laboratory, it was shown that i.m. electrotransfer of a plasmid encoding the influenza hemagglutinin induces a better immune response in mice than a single i.m. injection [121]. And recently, we have assessed the potential of i.m. electrotransfer in mouse to produce neutralizing antibodies, with high titer, against botulinum toxins, the most powerful poison in the world in present time [74]. We have optimized DNA electrotransfer for genetic immunization against botulinum antigen. This DNA immunization has been used in rabbits to induce antibodies production which is compatible with industrial development of antiserum production for a human therapeutic use (Burgain *et al.*, unpublished results). These examples show that it is possible to obtain high titers neutralizing antibodies in animals by DNA electrotransfer.

Monoclonal antibodies are increasingly being used in a wide range of clinical applications in the field of autoimmune disease, cancer and infectious disease. The production and secretion by electrotransferred muscle of monoclonal antibodies has been demonstrated by our group and the one of I. Mathiesen, independently [83, 122]. These studies demonstrated that the co-transfection of two naked plasmids encoding the heavy and light G immunoglobulin chains led to the secretion of fully assembled and functional immunoglobulin molecules. The successful neutralization of various pathogens resulted from monoclonal antibody secretion by electrotransferred muscle, raising the possibility of clinical passive immunization applications.

7. Conclusion

In vivo electrotransfer is a non-viral technique which has emerged as an efficient, user-friendly and cheap gene transfer method which issued for a wide range of tissues and species. Moreover, *in vivo* electrotransfer can be used for either local or distal effect by secretion of the transgenic protein into the bloodstream. The skeletal muscle is able to produce functional proteins with adequate post-translational modifications, which means that the muscle can be used as an endocrine organ for the production of therapeutic secreted proteins targeting systemic diseases. It is now established that therapeutic levels of circulating proteins can be reached in animal models. And since DNA does not induce any immune response, plasmid electrotransfer can be repeated as often as desired (Scherman *et al.*, unpublished results).

The understanding of the precise mechanism of electrotransfer, the optimization of its realization, the improvement of plasmids and of the structure of the encoded protein will bring more efficiency and above all more safety to the method, should it be applied to humans. Several clinical trials have been conducted and/or are still in progress. For more details see <http://www.clinicaltrials.gov/ct2/results?term=electroporation>. These clinical trials are mainly conducted against infectious diseases such as AIDS, hepatitis B, malaria, dengue, influenza... and various cancer types such as ovarian cancer or renal cancer, melanoma, cancers caused by human papillomavirus... Thus, DNA electrotransfer appears as a powerful and promising tool not only for gene therapy, but also for *in vivo* gene delivery at the laboratory level within the frame of physiological studies.

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siRNA and Gene Formulation for Efficient Gene Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Whilst small interfering RNA (siRNA, also known as short interfering RNA) has a somewhat chequered history with regard to its discovery and initial usage, the “mammalian” research community singularly neither reading nor citing the output from the “plant” research community, it is now recognised in terms of \$bn being invested and spent that RNA interference (RNAi), sequence specific post-transcriptional gene silencing (PTGS) by siRNA, has many potential therapeutic applications [1] as well as being an important tool in the study of functional genomics. The site and mechanism of action of siRNA requires that these short double-stranded nucleic acids are delivered to the cytosol of target cells. Therefore, formulation is required in a strategy similar to that for gene therapy, although not requiring access to the nucleus. Efficient medicines design should come with an understanding of the problem at the molecular level. Our contributions are aimed at the use of non-viral gene therapy and this Chapter therefore has such a focus.

2. RNA interference

2.1. History and mechanism of RNA interference

siRNA is a double-stranded RNA (dsRNA) typically of 21-25 nucleotides per strand. siRNA operates as a part of the cellular mechanism called RNAi, which was first noticed in petunia flowers (*Petunia hybrida*) which showed reduced pigmentation on the introduction of exogenous genes that were meant to increase pigmentation [2, 3]. These experiments aimed at increasing the pigmentation of the petunia flowers by means of introducing additional gene constructs expressing either chalcone synthase [2, 3] or dihydroflavonol-4-reductase [2]. However, the resultant plants produced completely white flowers and/or flowers with white

or pale sectors on a pigmented background. The exact mechanism was not identified at the time and was simply termed co-suppression. The transcription level of the suppressed chalcone synthase genes in petunia flowers was found to be similar to that of the non-suppressed genes, and thus the co-suppression must have been at the post-transcriptional level [4]. Later in 1997, the suppression of chalcone synthase endogene in petunia flowers was suggested to be related to formation of RNA duplexes by intermolecular pairing of complementary sequences between the coding sequence and the 3'-UTR sequence of the transgene mRNA [5]. Indeed, the seminal contributions the plant RNAi community have made to this RNAi field are also reflected in the research of Hamilton and Sir David C. Baulcombe in the Sainsbury Laboratory, Norwich, UK, on PTGS as a nucleotide sequence-specific defence mechanism that can target both cellular and viral mRNAs with RNA molecules of a uniform length, ~25 nucleotides [6]. That RNA silencing involves the processing of dsRNA into 21-26 long siRNA to mediate gene suppression (correspondingly complementary to the dsRNA) was demonstrated in *Arabidopsis*, "RNA silencing pathways in plants that may also apply in animals" [7]. That *Arabidopsis* ARGONAUTE1 RNA-binding protein is an RNA slicer that selectively recruits microRNAs and siRNAs was shown to be by a key mechanism similar to but different from that found in animals [8]. In 1998, Fire, Mello and co-workers reported the reduction or inhibition (hence genetic "interference") of the expression of the *unc-22* gene in *Caenorhabditis elegans* by means of dsRNA that is homologous to 742 nucleotides in the targeted gene [9], a discovery that was awarded the Nobel Prize in medicine or physiology in 2006. The target gene expresses an abundant although nonessential myofilament protein. Decreasing *unc-22* activity resulted in an increasingly severe twitching phenotype, while complete inhibition resulted in impaired motility and muscle structural defects. The target gene inhibition was best achieved with dsRNA, while using the individual sense or anti-sense RNA strands resulted only in modest silencing. The authors also noticed that only few copies of the dsRNA are required per cell to initiate a potent and specific response, rejecting the hypothesis that the mechanism of interaction with target gene mRNA is stoichiometric in nature, and thus the role of the dsRNA in the interference machinery must be catalytic or amplifying.

Elbashir et al. reported in 2001 that sequence-specific gene silencing of endogenous and heterologous genes with 21 nucleotide siRNA occurs in mammalian cell cultures [10]. The reporter genes coding for sea pansy (*Renilla reniformis*) and firefly (*Photinus pyralis*) luciferases were silenced successfully in different cell lines including human embryonic kidney cells (293) and the cervix cancer cells (HeLa cell line, the first human cell line grown in vitro with success [11]), as well as the endogenous gene coding for the nuclear envelope proteins lamin A and lamin C in HeLa cells. The authors used dsRNA of length 21 or 22 nucleotides with 3'-symmetrical 2-nucleotide overhangs on each strand, as dsRNA with length >30 nucleotides initiates an immune response e.g. inducing interferon synthesis) that leads to non-specific mRNA degradation, which was evident from non-specific silencing of luciferase with 50 and 500 nucleotides dsRNA in HeLa S3 cells, COS-7 cells (kidney cells of the African green monkey), and NIH/3T3 cells (mouse fibroblasts) [10]. The RNAi mechanism of action continues to be investigated in detail and reviewed thoroughly [12-17]. The RNAi mechanism involves the incorporation of dsRNA segments (e.g. siRNA) that have a sequence complementary to the targeted mRNA in a protein com-

plex. This core complex which carries-out mRNA degradation is the RNA induced silencing complex (RISC) [18-20]. The degradation process requires the key argonaute family of proteins, which contain a domain with RNase H (endonuclease) type of activity that catalyse cleavage of the phosphodiester bonds of the targeted mRNA. RISC assembly and subsequently its function to mediate sequence specific mRNA degradation occur in the cytoplasm of the cell [16]. The source of the dsRNA segments incorporated in RISC can be endogenously processed microRNA (miRNA), short hairpin RNA (shRNA), or synthetic siRNA. miRNA is produced from endogenous DNA through the action of RNA polymerase II resulting in the formation of non-coding RNA called primary miRNA (pri-miRNA), which is processed in the nucleus by a protein complex containing an enzyme known as Drosha and a dsRNA binding protein cofactor called Pasha (DGCR8). Drosha cleaves pri-miRNA to produce (pre-miRNA), a dsRNA of 70-90 nucleotides and having a hairpin loop, which binds to Exportin 5 protein and is transferred from the nucleus into the cytoplasm. Pre-miRNA is processed by Dicer (RNase III enzyme) in the cytoplasm to give miRNA, typically of 22 nucleotides in length and having two nucleotide overhangs at the 3'-position [16, 21], shRNA is produced by transcription from an exogenous DNA that is delivered to the nucleus, and codes for a hairpin shaped RNA with segments of length 19-29 nucleotides and loop of 9 nucleotides [22, 23] which can then be processed by Dicer and incorporated in the RNAi machinery.

Once in the cytoplasm, the processed dsRNA (miRNA, processed shRNA, or siRNA) is then incorporated into a protein complex (RISC-loading complex, RLC). In *Drosophila* the RLC is composed of the dsRNA, heterodimer protein DCR2 (Dicer variant)/R2D2, possibly including the catalytic argonaute proteins as well in this complex. The active RISC is formed when one of the RNA strands in the complex is cleaved (the passenger strand) and the strand with the less thermodynamic stable 5'-end (guide/anti-sense strand) remains in the complex. The mRNA with complementary sequence to the guide strand binds to the active RISC and is cleaved by the endoribonuclease activity of the argonaute component of the complex (Figure 1).

2.2. RNA duplex structure

RNA is a polymer of ribonucleotides. Each RNA nucleotide is composed of one nucleobase, the monosaccharide pentose ribose, and one phosphate group. The nucleobases in RNA are adenine (purine base), guanine (purine base), uracil (pyrimidine base), and cytosine (pyrimidine base) (Figure 2). A nucleoside is formed when each base is connected via a glycosidic bond to the anomeric carbon 1' of ribose, thus when glycosylated, adenine, guanine, uracil, and cytosine nucleobases give adenosine, guanosine, uridine, and cytidine nucleosides. Each two nucleosides are connected via a phosphate diester bond between the 3' of one nucleoside and 5' of the next nucleoside to form the RNA polynucleotide strand. The main differences in the primary structure of RNA and DNA are that RNA pentose is ribose while DNA pentose is 2'-deoxyribose, and the RNA incorporates the nucleobase uracil instead of thymine.

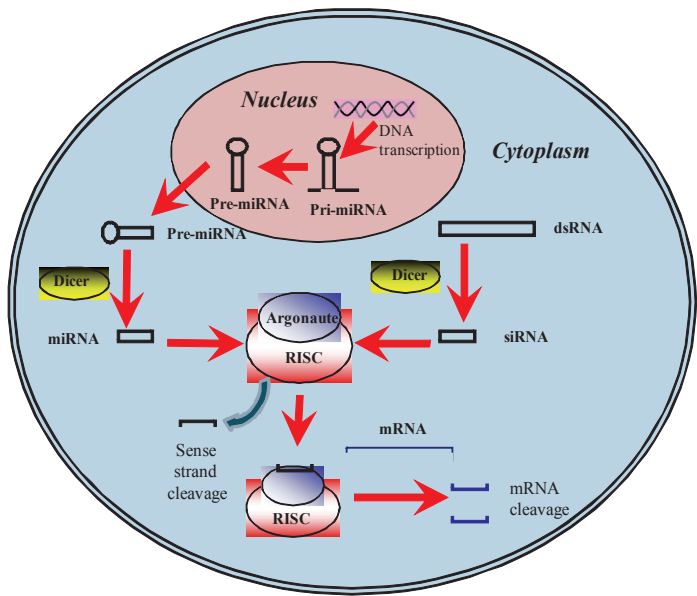


Figure 1. RNAi mechanism in a eukaryotic cell. The source of the antisense strand incorporated in RISC can be miRNA, processed exogenous long dsRNA, or synthetic siRNA delivered to the cell.

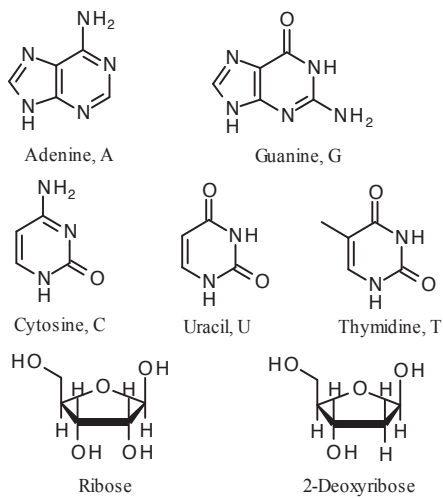


Figure 2. Nucleobases and pentoses of RNA and DNA.

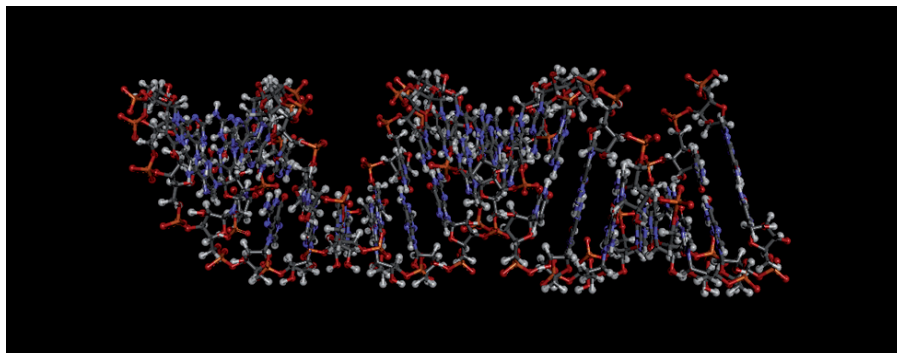


Figure 3. siRNA duplex 22-mer targeting the enhanced green fluorescent protein (EGFP) mRNA. The two deoxythymidine residues at the 3'-end of the sense strand are not shown. Sense strand: 5'-GCAAGCUGACCCUGAAGUUCAUTT-3' Anti-sense strand: 5'-AUGAACUUCAGGGUCAGCUUGCCG-3' Target DNA sequence: 5'-CGGCAAGCTGACCCTGAAGTTCAT-3'

In order to form an RNA duplex (Figure 3), the strands with complementary nucleotide sequence bind together by hydrogen bonds. Adenine is bound to uracil with two hydrogen bonds while guanine is bound to cytosine with three hydrogen bonds, thus forming what is known as Watson-Crick base pairs. RNA duplexes under normal physiological conditions are in the form of A-helix. This type of duplex is a right-handed helix [24-26].

The presence of the 2'-hydroxyl group of the ribose and the lack of the methyl group on the nucleotide uridine (in contrast to the methylated thymidine) results in structural differences between RNA and DNA, with the 2'-hydroxyl group of RNA being the major cause of the differences. The sugar phosphate backbone of RNA duplexes is stabilized by the 2'-hydroxyl in the C3'-endo position, while DNA adopts the C2'-endo position (Figure 4). Thus, the RNA duplex takes the A-helix form while the DNA helix takes the B-form. The A-helix form is suggested to have a greater hydration shell, giving RNA duplexes more thermodynamic stability and more rigidity compared to DNA duplexes [24-26]. RNA A-helix completes one complete rotation in 11-12 base pair (bp) compared to 10 bp for DNA, with a rise of 2.7 Å per bp of RNA [27]. The A-helix geometry has been suggested to be the major factor explaining why dsRNA and not dsDNA is involved in the RNAi machinery [28], where the A-helix geometry between the guide strand and the complementary target mRNA is essential for the catalytic activity of the argonaute 2 protein in the RISC.

As a result of the presence of a hydroxyl group in the 2'-position of the ribose in the RNA backbone, the RNA phosphodiester backbone is more susceptible to hydrolysis by nucleases compared to the DNA which lacks the 2'-hydroxyl in its 2'-deoxyribose [29]. Incubation of

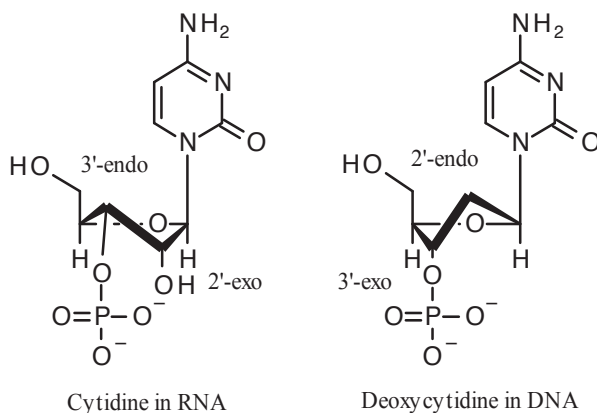


Figure 4. 3'-endo ribose configuration of RNA (left) vs 2'-endo (right) of 2'-deoxyribose in DNA. Shown is cytidine (RNA) and deoxycytidine (DNA) with the 3'-hydroxyl phosphorylated. The hydrogen atoms at C2' and C3' are not displayed for clarity.

siRNA in fetal bovine or human serum at 37 °C resulted in the degradation and partial or complete loss of activity [30]. When incubated in human plasma at 37 °C, more than 50% of the unmodified siRNA was degraded within one minute, and practically all siRNA was completely degraded within 4 hours [31]. Although Ribonuclease A (RNase A, an endoribonuclease) cleaves single stranded RNA, siRNA degradation in serum was reported to be mainly due to RNase-like activity[32], which is suggested to occur during transient breaking of the hydrogen bonds joining the two siRNA strands. In addition to the RNase A family of enzymes, blood serum contains phosphatases and exoribonucleases which can also affect degradation of siRNA at nuclease-sensitive sites on both strands [33].

2.3. Therapeutic potential of RNAi based therapies

RNAi based therapies emerged in the period following its discovery in 1998 in plants, and are promising therapeutic candidates to treat various types of diseases, ranging from age related macular oedema to respiratory tract infections to various types of cancer [34-36]. In addition to siRNA based therapies, shRNA [37, 38] and miRNA [39] are potential therapeutic tools. siRNA based therapeutics are already in phase I and phase II clinical trials; representative examples of clinical trials involving siRNA are shown in Table 1. The basic concept is the reduction or inhibition of the expression of a protein that is involved in the pathophysiological pathway of the target disease (silencing/knocking-down the target gene). This concept is evident from using Cand5 siRNA targeting the mRNA translating the vascular endothelial growth factor (VEGF), thus reducing/inhibiting angiogenesis and preventing progression of wet age related macular oedema (Table 1) [40]. Atu027 siRNA targets the biosynthesis of protein kinase N3 which plays a role in cancer metastasis [41].

siRNA	Disease	Vector/ Route	Phase	Sponsor
Cand5/ Bevasiranib	Diabetic macular oedema	None/ <i>Intravitreal</i>	Phase II	Opko Health (Miami, USA)
Cand5/ Bevasiranib	Age-related macular degeneration	None/ <i>Intravitreal</i>	Phase II (Phase III halted)	Opko Health (Miami, USA)
ALN-RSV01	Respiratory syncytial virus infection	None/ <i>Intranasal</i>	Phase II	Alnylam Pharmaceuticals (Cambridge, USA)
CALAA-01	Solid tumour/melanoma	Cyclodextrin nanoparticles/ <i>Intravenous</i>	Phase I	Calando (Pasadena, CA, USA)
Atu027	Colorectal cancer metastasizing to the liver	AtuPlex- Liposome/ <i>Intravenous</i>	Phase I	Silence Therapeutics (London, UK)
Two siRNA against TGFBI and COX-2 STP705	Wound healing	Nanoparticles/ <i>Intravenous</i>	Phase I	Sirnaomics (Gaithersburg, MD, USA)
ISNP	Protection from acute kidney injury after cardiac bypass surgery	None/ <i>Intravenous</i>	Phase I	Quark Pharmaceuticals (Fremont, USA)
TKM-080301	Against PLK1 gene product in patients with hepatic cancer	Lipid nanoparticles/ <i>Hepatic intra- arterial administration</i>	Phase I	NCI (Maryland, USA)

Table 1. Representative clinical trials using siRNA (<http://clinicaltrials.gov/ct2/home>, accessed on 5/8/2012).

The therapeutic application of siRNA requires overcoming several barriers (Figure 5) for its intracellular delivery and the subsequent functional gene silencing activity [42-44]. Those barriers are mainly due to siRNA specific characteristics, most important are having a highly negative charge due to their phosphate backbone (on average 40-50 negative charges per siRNA), being susceptible to degradation by nucleases, and having relatively large molecular weight (13-15 kDa) compared to conventional small drug molecules. First, local delivery (such as intravitreal) is different from intravenous delivery, where the latter will subject the siRNA to the serum ribonucleases, which results in degrading non-modified siRNA within time periods that vary from minutes to hours [31]. siRNA injected intravenously in rats was reported to be cleared rapidly from circulation and accumulates in kidneys within minutes of injection [45], making it useful only if the target organ is the kidney.

In order to gain access into the cytoplasm where siRNA can exert its biological activity, the polyribonucleotide must pass first through the interstitial space then through the target cell membrane. This will be a difficult task, since both the extracellular matrix in many tissue types

and the cell membrane incorporate negatively charged glycosaminoglycans (e.g. heparan sulfate) [46]. In addition, cell membranes contain negatively charged phospholipids (e.g. phosphatidyl serine) therefore the membrane is negatively charged [46, 47]. The net result is an unfavourable repulsive interaction with naked siRNA. As a result, different strategies are being developed to overcome the barriers to reproducible and functional siRNA delivery, and these approaches fall into two general categories. One category is modifying the siRNA, the other is deploying a vector to protect the siRNA and increase its efficiency of delivery.

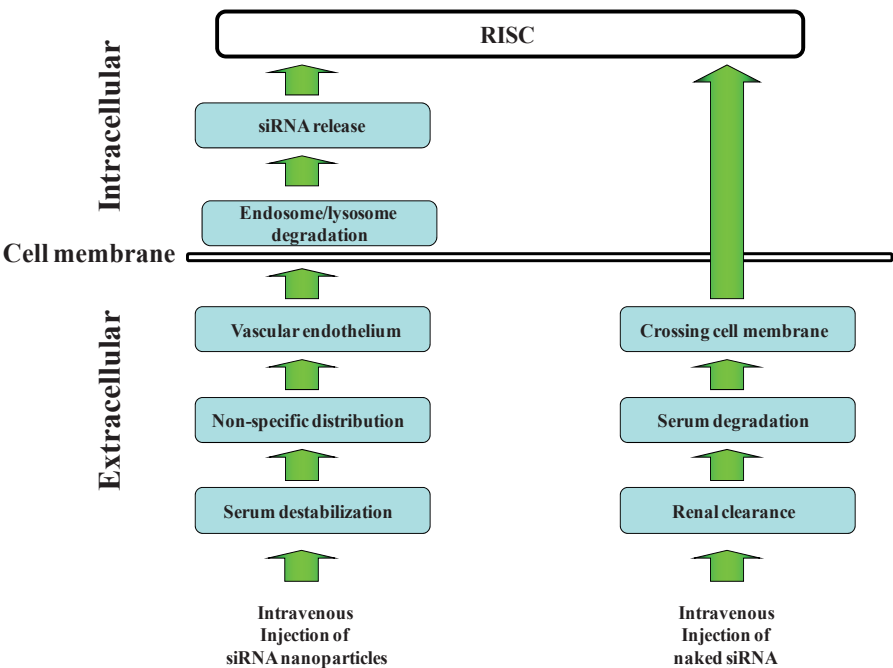


Figure 5. Summary of barriers to successful gene-silencing mediated by siRNA after intravenous injection, whether delivered naked or incorporated in nanoparticles.

3. Strategies to achieve efficient siRNA delivery and gene silencing

3.1. siRNA modifications

siRNA modifications include those carried out at the ribose residue, at the phosphate backbone, at the RNA nucleotides, the siRNA termini, and/or by conjugation of other molecules to the siRNA molecule. Modifications to the ribose at the 2'-position are common [48], and include 2'-O-alkylation (e.g. 2'-O-methyl and 2'-O-methylethoxy) modifications. 2'-Fluoro RNA is

another common modification. Locked nucleic acids (LNAs) have a methylene bridge connecting the 2'-O to the 4'-C of the ribose unit, locking the sugar in the 3'-endo conformation. These modifications led to increased ribonuclease resistance [48, 49]. Modifications at the phosphate backbone include phosphorothioate, boranophosphate, and methylphosphonate linkages [48, 49] and is reported to increase siRNA stability against various ribonucleases and phosphodiesterases [50]. siRNA nucleotides can be substituted with DNA nucleotides to increase stability and/or decrease unwanted siRNA off-target effects [51]. Modifications of the 3'-overhangs (usually two nucleotides in length) include incorporating deoxyribonucleotides to reduce costs and increase stability towards 3'-exoribonucleases. The 5'-terminus chemical phosphorylation of the antisense strand results in higher gene silencing efficiency, while blunt ended duplexes were reported to be more resistant to exonucleases. The advantages of each of the aforementioned techniques, other modification strategies, as well as the considerations related to the degree of modification and its effect on gene silencing efficiency and associated cytotoxic effects have been reviewed thoroughly [48, 52-54].

The conjugation of drug molecules, aptamers, lipids, polymers, and peptides/proteins to siRNA could enhance in vivo delivery [55]. The main aims of such conjugations are: to enhance siRNA stability, increase in vivo half-life, control biodistribution, increase efficiency of intracellular delivery, while maintaining the gene silencing activity.

One strategy is to increase the hydrophobicity of the siRNA. Cholesterol was conjugated to the 5'-terminus of siRNA, the cholesterol-siRNA conjugate (chol-siRNA) resulted in better intracellular delivery compared to unmodified siRNA and retained gene silencing activity in vitro in β -galactosidase expressing liver cells [56]. When cholesterol was conjugated to the 3'-terminus of the sense (passenger) strand of siRNA, the conjugate had improved in vivo pharmacokinetics as the intravenous administration of chol-siRNA in mice resulted in its distribution and detection in the fat tissues, heart, kidneys, liver, and lungs, even 24 h after intravenous injection [57]. No significant amounts of unmodified siRNA were detected in the tissues 24 h after the intravenous injection. Conjugation of siRNA to bile acids and long-chain fatty acids, in addition to cholesterol, mediates siRNA uptake into cells and gene silencing in vivo [58]. The medium chain fatty-acid conjugates, namely lauroyl (C12), myristoyl (C14) and palmitoyl (C16), did not silence the target apolipoprotein B mRNA levels in mouse livers after intravenous injection. However, siRNA fatty-acid conjugates having long saturated chains, stearoyl (C18) and docosanoyl (C22), significantly reduced apolipoprotein B mRNA levels.

Cell penetrating peptides (CPPs) are used to facilitate cellular membrane crossing of many molecules displaying various properties such as antisense oligonucleotides, peptides, and proteins and are already being tested in vivo [59]. siRNA was conjugated to penetratin and transportin, to silence luciferase and green fluorescent protein (GFP) in different types of mammalian cells [60]. However, in vivo lung delivery in mouse of siRNA conjugated to penetratin and TAT(48-60), targeting p38 MAP kinase mRNA showed that the reduction in gene expression was peptide induced and the penetratin conjugated siRNA resulted in innate immunity response [61].

siRNA functioning against the VEGF mRNA was conjugated to poly(ethylene glycol) (PEG, 25 kDa) via a disulfide bond at the 3'-terminus of the sense strand [62]. The siRNA-PEG

conjugate formed polyelectrolyte complex (PEC) micelles by electrostatic interaction with the cationic polymer polyethylenimine (PEI). The formed VEGF siRNA-PEG/PEI PEC micelles showed enhanced stability against nuclease degradation compared to the unmodified siRNA. These micelles efficiently silenced VEGF gene expression in prostate carcinoma cells (PC-3) and showed superior VEGF gene silencing compared to VEGF siRNA/PEI complexes in the presence of serum. PEG conjugation on its own enhanced the stability of the siRNA in serum containing medium. The prolonged stability of the PEC micelles was suggested to be due to the presence of PEG chains in the outer micellar shell layer, thus sterically hindering nuclease access into the siRNA in the micelle core [62]. Targeting molecules such as antibodies [63] and aptamers (peptides or single stranded DNA or RNA that have selective affinities toward target proteins) [64] have also been conjugated to siRNA, with the aim of increasing the efficiency of siRNA delivery to the target tissues.

Conjugating molecules to siRNA requires specific considerations. First, the site of conjugation (3'- and/or 5'-terminus, on sense and/or antisense strand) should be chosen such that it does not affect the activity of the siRNA and its ability to be incorporated in the RISC, or its ability to bind the target mRNA in the correct helix conformation. Second, the conjugated siRNA might have new properties that were not present in the unmodified parent siRNA. An example is the *in vivo* immune response resulting from the penetratin-siRNA conjugate [61]. Third, the conjugation process is multi-step, and the chemical reaction intermediates and products require efficient purification in order to meet the specifications of *in vivo* applications. These steps need to be repeated for each siRNA under investigation, which can be costly and time consuming. Thus, although there are clear advantages to synthesize siRNA conjugates, there are also disadvantages, and conjugation is therefore only one of two valuable approaches in the toolbox for preparing siRNA based therapies. The other valuable tool is complexation or incorporating the siRNA in a vector.

3.2. Viral vectors for shRNA delivery

Vectors for RNAi based therapies are either viral or non-viral vectors. Viral vectors (Table 2) are used to deliver genes encoding hairpin RNA structures such as shRNA and miRNA, which are then processed by the cellular RNAi machinery to the functional silencing dsRNA [65, 66].

Viral vectors offer two main advantages, the first is the very high efficiency compared to non-viral vectors [68], which can reach few orders of magnitude more than that achieved with non-viral vectors, and the second is the potential of long term expression of the delivered RNAi therapeutic, which is very useful in the treatment of chronic diseases such as HIV infection and viral hepatitis [69, 70]. Retroviruses are enveloped, single stranded RNA viruses and have a genome capacity of 7-10 kilobases (kb). They preferentially target dividing cells which limits their use to mitotic tissues (thus for example excluding brain and neurons). Retroviruses integrate their DNA in the host genome using an integrase enzyme, which provides the advantage of stable long term expression of the delivered transgene in the host cell and its descendants. However, integrating new DNA sequences into host genome carries the risk of

	Retrovirus/ Lentivirus	Adenovirus	Adeno- associated virus	Herpes virus
Viral vector properties	Genome	ssRNA	dsDNA	dsDNA
	Capsid	Icosahedral	Icosahedral	Icosahedral
	Envelope	Enveloped	None	Enveloped
	Viral Polymerase	Positive	Negative	Negative
	Diameter (nm)	80-130	70-90	18-26
	Genome size (kb)	7-10	38	5
Gene therapy related	Infection tropism	Dividing*	Dividing/ Non-dividing	Dividing/ Non-dividing
	Virus genome integration	Integrating	Non-integrating	Integrating
	Transgene expression	Lasting	Transient	Lasting
	Packaging capacity (kb)	7-8	8	4.5
				>30

* Lentiviral vectors can infect non-dividing cells as their pre-integration complex can traverse the nuclear membrane pores (NMP), in contrast to retrovirus pre-integration complex which does not traverse NMP, requiring the host-cell division to integrate the retroviral genome [67].

Table 2. Summary of properties of viral vectors that are commonly used in gene therapy (adapted from <http://www.genetherapy.net/viral-vectors.html>, accessed on 5/8/2012).

insertional mutagenesis [68, 71]. shRNA expression cassette delivered by a retroviral vector was used in rats to silence a RAS oncogene in order to suppress tumour growth [72]. Herpes virus was used successfully to deliver shRNA targeting exogenous β -galactosidase or endogenous trpv1 gene mRNA in the peripheral neurons in mice by injecting once directly into the sciatic nerve of the animals [73].

Unlike other retroviruses, lentiviruses can infect dividing as well as differentiated and non-dividing cells. The lentiviral genome can accommodate 7.5 kb [66], and their genome is integrated in the host cell genome, lentiviral vectors are generally preferred for long-term expression of transgenes, and efficient delivery in vivo to the brain, eye, and liver to induce long-term transgene expression as reported [74]. A lentiviral vector was used to deliver shRNA targeting *Smad3* gene mRNA, and enhanced myogenesis of old and injured muscles [75].

Adenoviruses are non-enveloped viruses, with linear double stranded DNA. They preferably infect the upper respiratory tract and the ocular tissue. Their genome can accommodate up to 8 kb which can be extended to ≥ 25 kb in modified viruses that have their viral genes deleted [68]. These viruses can infect post mitotic cells and thus are good candidates for neurological diseases. Unless delivering genes that can exist as episomes in host cells, adenoviruses result only in transient expression of their cargo. However, although the host cells with the episome can express the delivered genes for the cell life time, these cells will eventually be removed by the host immune system [68]. shRNA targeting VEGF that was delivered by an adenoviral vector resulted in potent inhibition of angiogenesis and tumour growth in mice [76].

Adeno-associated virus (AAV) is a single stranded DNA non-pathogenic virus that can accommodate a 4.7 kb genome. They can infect dividing or non-dividing cells. The replication of AAV requires co-infection with adenovirus. The viral genome integrates into the host cell genome at a specific location on chromosome 19 [68]. Direct intracerebellar injection in a mouse model of spinocerebellar ataxia of an AAV vector delivering a cargo expressing shRNA targeting polyglutamine induced neurodegeneration significantly restored cerebellar morphology and improved motor coordination in mice [77].

Although highly efficient in delivering their cargo, viral vectors have their disadvantages. Adenoviral vectors have the disadvantage of triggering a strong immune (adaptive and innate) response by repeated administration, in addition to target organ immunotoxicity, specially hepatotoxicity [78-80], which resulted in 1999 in the death of one 18-year-old male who received high dose of adenovirus that was delivered directly in the hepatic artery in a clinical gene therapy safety study [81]. Clonal T-cell acute lymphoblastic leukemia caused by insertional mutagenesis in a gene therapy completed clinical trial involving patients suffering X-linked severe combined immunodeficiency (SCID-X1) was reported in one out of the 10 patients using a retroviral vector [82]. Integration of the vector genome material in the antisense orientation 35 kb upstream of the protooncogene (LMO2) caused over expression of the gene in the leukemic cells. In a similar study, 4 out of 9 patients developed leukemia within 3-6 years post-treatment mainly due to vector-mediated upregulation of host cellular oncogenes [83, 84]. In addition, immune responses (whether adaptive or innate) of varying degrees depending on the type of vector, dose, and target organs were reported for lentiviral, adenoviral, adeno-associated viral vectors [80].

Current research on viral vectors for gene therapy is focussed on approaches such as vector engineering e.g. modifying the viral capsid or pseudotyping the envelope, different delivery strategies, and administration to immune-privileged sites that can tolerate the delivered viral vectors without responding with an inflammatory response [80, 85]. Other research focusses on the essential scaling-up process of vector production and increasing the packaging efficiency of the vectors [85], the processes without which, the wide spread and successful therapeutic use of the viral vectors will be very difficult to achieve.

3.3. Non-viral vectors

Non-viral vectors for gene and siRNA delivery are an alternative to viral vectors, as they do not suffer many of the disadvantages of the viral vectors, especially immunogenicity and tumourigenicity. The non-viral vectors can be classified generally as peptides, polymeric based vectors, carbohydrate based, and lipid based [86]. CPPs, also known as peptide transduction domains (PTDs), have shown the ability to cross the cellular membrane despite their relatively high molecular weight and size (Table 3).

PTDs generally are short amphipathic and/or cationic peptides that can transport many hydrophilic molecules across the cell membrane. A wide range of molecules including liposomes [87, 88], peptides, proteins [89], peptide nucleic acids [90] and polynucleotides [91] are delivered intracellularly using PTDs and they have also been applied in vivo [59, 92, 93].

CPP	Sequence of CPP	Type of association with siRNA	Target mRNA
CADY	GLWRALWRLSLRWLLWRA	Non-covalent	GAPDH, p53 [94]
EB1	LIRLWSHLIHWQNRRLKWKKK	Non-covalent	Luc [95]
MPG	GALFLGFLGAAGSTMGAWSQPKKRRKV	Non-covalent	Luc, GAPDH [96] Oct-3/4 [97]
Poly-arginine	RRRRRRRRR	Non-covalent	VEGF [98]
Penetratin	RQIKIWFQNRRMKWKK	Covalent	Luciferase (Luc), EGFP [60]
		Covalent	SOD1, caspase-3 [99]
		Covalent	Luc, p38 MAP kinase [61, 100]
Transportan	LIKKALAAALAKLNILLYGASNLTWG	Covalent	Luc, EGFP [100]
TAT	GRKKRRQRRPPQ	Covalent	EGFP, CDK9 [101]

Table 3. Selected CPPs used for siRNA delivery [59].

It was reported by Frankel and Pabo in 1988 that the HIV-1 derived TAT protein could be taken up by cells growing in tissue culture [102], and that a small basic region of TAT (48-60) was essential for uptake by the cells [103]. PTDs include antennapedia homeodomain protein (Antp, penetratin), mitogen-activated protein (MAP), poly-arginine, transportan, VP22 [59, 92]. Two major pathways are involved in the uptake of PTDs and PTD-cargos: direct translocation at 4 °C and 37 °C and endocytosis-translocation at 37 °C. These mechanisms depend on many factors: cargo size, cell line, PTD concentration, and the type of PTD [59, 104, 105]. siRNA can be conjugated covalently to the CPP or can be complexed with the cationic groups of basic amino acids that are present in the backbone of the CPP. As a representative example of non-covalent complexation, CADY [94], which is basic due to its five arginine residues can complex with the negatively charged siRNA. Another example of non-covalent complexation is the poly-arginine CPP [98].

PEI (Figure 6) is an efficient, but toxic, plasmid DNA delivery vector. However, as a siRNA delivery vector PEI is reported to be much less efficient [106, 107]. This decreased efficiency is due to the dissociation of the siRNA/PEI complex upon interaction with the negatively charged cell membrane, which is suggested to be because of the short length of siRNA and the associated weak electrostatic interaction with PEI [108, 109]. Another drawback of PEI is its relatively high toxicity [110]. Thus, in addition to linear PEI, PEI polymers with a wide range of molecular weights were developed to increase PEI efficiency and/or decrease toxicity, although not all PEI are suitable for siRNA delivery [111]. The main advantage of PEI is the ability of its variety of amino groups to be protonated at lower pH (inside endosomes) leading to what is known as the “proton-sponge effect” [112], and efficient escape of the nucleic acid cargo from endosomes.

One approach to enhance siRNA delivery with PEI is increasing the hydrophobicity of PEI by covalently conjugating alkyl chains [113], where increasing the hydrophobic alkyl chain length generally improved the stability of the PEI/siRNA complex. In a similar strategy, cholesterol was conjugated to PEI with decreased toxicity of the conjugates [114]. Low molecular weight PEI (MW < 5 kDa) is less toxic than the higher molecular weight PEI (≈25 kDa), but less efficient

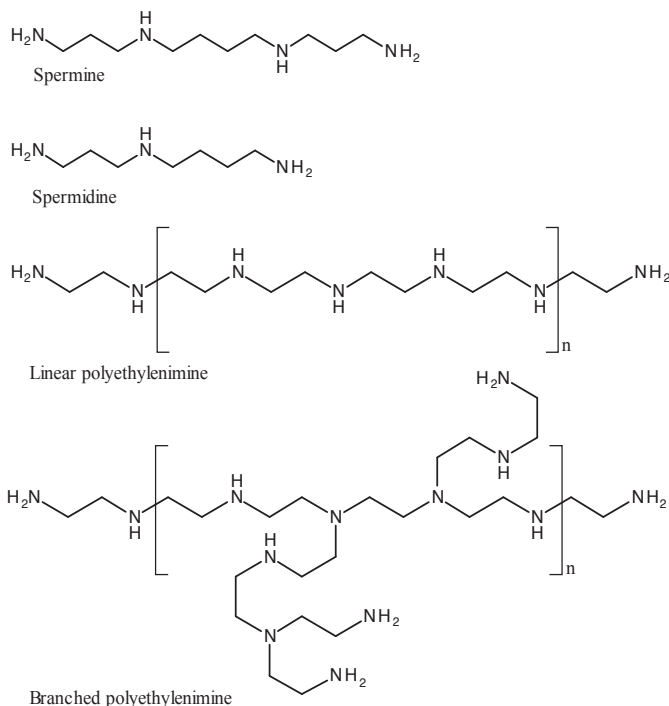


Figure 6. Representative examples of polyamines used in siRNA delivery either as in lipid conjugates of polyamine alkaloids e.g. spermine and spermidine, or as in polyethylenimine (PEI), a cationic polymer.

in polynucleotide delivery, thus, cross-linking of the low molecular weight PEI with disulfide bonds which are cleaved in the reducing environment of the cytoplasm increased the efficiency of siRNA delivery through the enhanced release of siRNA in the cytoplasm [115].

Chitosan is a biocompatible and biodegradable polysaccharide that is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. Chitosan has weakly basic properties due to the presence of the D-glucosamine residue with a pK_a value 6.2-7.0. The molecular weight of chitosan affects the complex stability, size, zeta-potential and in vitro gene knock-down of siRNA/chitosan nanoparticles [116]. High molecular weight (64.8-170 kDa) chitosan formed stable complexes with siRNA and resulted in high gene knock-down efficiency in human lung carcinoma (H1299) cells, while low molecular weight (10 kDa) chitosan could not complex the siRNA into stable nanoparticles and showed almost no knock-down [117]. The method of association affects gene silencing efficiency, where chitosan-TPP/siRNA nanoparticles (siRNA entrapped inside the nanoparticles, and TPP is sodium tripolyphosphate and used as a polyanion to cross-link with the cationic chitosan groups by electrostatic interactions) showed high siRNA binding and better gene silencing in vitro compared to siRNA/chitosan particles prepared by

simple complexation and adsorption of siRNA onto chitosan [118]. Although chitosan has good potential as a non-viral gene delivery vector, widespread use is largely limited by its poor solubility (because of their pKa, chitosan amino groups are only partially protonated at the physiological pH 7.4), poor stability of its siRNA complexes at the physiological pH, and low transfection efficiency. Various strategies have been adopted to overcome these drawbacks, such as covalently conjugating PEG to chitosan and binding targeting ligands to enhance cell specificity [116].

Cyclodextrins (CD) are cyclic oligosaccharides composed of 6, 7, or 8 D-(+)-glucose units, known as α -CD, β -CD, γ -CD respectively, bound through α -1,4-linkages. Polymers conjugated to β -CD lack immunogenicity and hence are attractive vectors for polynucleotide delivery. β -CD have a hydrophilic outer surface and a hydrophobic inner cavity which enable them to form inclusion complexes. Efficient cellular transfection of siRNA labelled with a fluorescent tag into human embryonic lung fibroblasts (MRC-5 cells) was observed by siRNA complexes with the β -CD guanidine derivatized bis-(guanidinium)-tetrakis-(β -cyclodextrin) tetrapod (having four β -CD units) [119]. The ability of β -CD to form inclusion complexes was used to develop a siRNA delivery vector. β -CD was covalently bound to a polycationic segment (to electrostatically bind siRNA), while adamantane-PEG-transferrin (adamantane can fit in the β -CD cavity) formed an inclusion complex which can enhance the stability of siRNA nanoparticles in vivo [120]. This system was used to deliver siRNA silencing the *EWS-FLI1* gene thus inhibiting tumour growth in a murine model of metastatic Ewing's sarcoma. The first experimental siRNA therapeutic to provide targeted delivery in humans was reported by Davis and co-workers [121]. siRNA was formulated into a nanoparticle (CALAA-01), which consisted of a cyclodextrin-containing polymer that contains amidine and primary amine functional groups, a PEG for steric stabilization in the in vivo environment (via inclusion complexes of β -CD with adamantane-PEG conjugate), and human transferrin (Tf) as the targeting ligand to binds to the transferrin receptors that are over-expressed on cancer cells. The siRNA/nanoparticles components self-assembled in the pharmacy. CALAA-01 was administered intravenously to the first patient with a solid cancer in a phase I clinical trial (safety study) in May 2008 [121]. Tumour biopsies from patients' melanoma after treatment (phase I clinical trial) showed the presence of intracellular nanoparticles. Reductions in the levels of both the specific mRNA (M2 subunit of ribonucleotide reductase, RRM2) and the protein (RRM2) were found when compared to levels in pre-dosing tissues. These results demonstrated that siRNA nanoparticles administered systemically to a human patient can produce a specific gene knock-down via an RNAi mechanism of action [122]. A recent and novel approach to the synthesis of cationic or neutral PEGylated amphiphilic β -CD used copper-catalysed "click" chemistry to modify selectively the secondary 2-hydroxyl group of the β -CD. The 6-position of these β -CD conjugates was conjugated to a dodecane alkyl chain. Complexation of cationic β -CD alone with siRNA resulted in good silencing of the luciferase reporter gene in Caco2 cells in culture. Co-formulation of cationic β -CD with a PEGylated β -CD and siRNA resulted in lower surface charges and reduced aggregation. The transfection efficiency of the cationic β -CD vector was lowered by co-formulation with the PEGylated β -CD, although the siRNA binding was not affected and the surface charge of the complexes did not reach complete neutrality [123].

Dendrimers have a central core to which are connected several branched arms in a manner that can be symmetrical or asymmetrical. During the synthesis of dendrimers, arms (branches) are added to the core structure. Each addition is called a generation and increases the previous generation number by one. Due to their unique structure, dendrimers can have a planar, elliptical, or spherical shape depending on generation number. Among the most widely used dendrimers are polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers [124]. Dendrimers which have positively charged cationic groups on their outer surface are commonly used for polynucleotide delivery. The transfection efficiency of dendrimers increases with increasing the charge density or generation number [125]. However, dendrimers with high generation number are generally more cytotoxic compared to dendrimers with low generation number [126]. Usually the inner space near the core is larger compared to outer space near the surface due to the lower density of molecules (less number of arms) near the core, which allow small molecules to be incorporated in the inner space. Owing to the relatively large molecular weight of polynucleotides, they are usually bound to the surface of cationic dendrimers and not in the inner space of the dendrimer. Generally, the toxicity of dendrimers is lower than that of PEI or poly-L-lysine (PLL) [127]. One advantage of dendrimers is that they have pH buffering capacity (proton-sponge effect), an important feature for endosomal escape and enhancing the release of polynucleotides [125, 128].

PPI dendrimers with high generation numbers (4 and 5) were more efficient in forming discrete nanoparticles with siRNA and in gene silencing in human lung cancer (A549) cells than lower generation dendrimers (2 and 3). Generation 5 PPI dendrimers were more toxic, probably due to the increased positive charge density per dendrimer, than generation 4 dendrimers [129]. Complex formation between PAMAM dendrimers with an ethylenediamine core and siRNA as a function of three variables has been reported [130]. The ionic strength of the medium (without or with 150 mM NaCl), the generation number (4, 5, 6 and 7) and the *N/P* ratio (ratio of positively charged amine groups per negative phosphate) were varied. The size of the complexes depended on the ionic strength of the media, with the strong electrostatic interactions in medium without NaCl making siRNA/dendrimer complexes smaller than those obtained in 150 mM NaCl. Both the intracellular delivery and the silencing of EGFP expression in cell culture was dependent on complex size, with smaller complexes efficiently delivered, and resulting in the highest silencing of EGFP expression. siRNA complexed with generation 7 dendrimers resulted in the highest silencing of EGFP expression both in human brain tumour cell line T98G-EGFP (35%) and mouse macrophage cell line J-774-EGFP (45%) cells, in spite of having lower protection of siRNA against degradation with RNase A, showing the importance of formulation procedures on the efficiency of transfection [130].

4. Cationic lipids as non-viral vectors for siRNA and DNA delivery

4.1. Gene delivery by cationic lipids

Gene delivery (DNA transfection) with cationic lipids (Figure 7) dates back to 1987 when it was reported by Felgner et al. [131], and the term “lipofection” was coined. Small unilamellar

liposomes containing the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl ammonium chloride (DOTMA) was reported to spontaneously complex DNA completely entrapping the DNA, and enhanced fusion with the cell membrane in vitro in cell cultures, resulting in efficient delivery and expression of the delivered DNA. The lipofection was 5-100-fold more effective than the commonly used transfection techniques at the time by either calcium phosphate or DEAE-dextran (diethylaminoethyl-dextran), depending on the cell line used [131]. Cationic lipids have polar and non-polar domains and thus are amphiphilic in nature, with three general structural domains: (a) a cationic hydrophilic head-group (positively charged). The head-group can carry a permanent positive charge as in quaternary ammonium groups, or can be protonated at the physiological pH 7.4, such as primary and secondary amine groups. There can be one cationic group per lipid molecule (monovalent cationic lipids) or more than one cationic group per lipid molecule (multivalent cationic lipids); (b) a hydrophobic domain covalently attached by a linker to the cationic head-group. This domain can be in the form of either alkyl chains (commonly 2 chains) of various chain lengths (with various oxidation states) or can be a steroid such as cholesterol; (c) the linker between the head-group and the hydrophobic domain [132, 133]. This linker controls the biodegradation of the cationic lipid and its stability under different conditions according to the type of chemical bonds (e.g. ester, ether, or amide). Each domain can be controlled to change a specific character of the cationic lipid, e.g. using a disulfide functional group as a bioresponsive linker [134] which is reduced in the intracellular environment by glutathione/glutathione reductase and enhance biodegradation characters of the lipid and decrease its cytotoxicity.

4.2. The cationic head-group

The cationic head-group's main function is to bind electrostatically the negatively charged phosphates of the polynucleotides. The complexes of cationic lipids with polynucleotides such as DNA and siRNA are called lipoplexes. This requires the presence of a positive charge on the head-group at the physiological pH 7.4, i.e. the pKa of the head-group is ideally at least one unit higher than the physiological pH. The most commonly used head-groups contain nitrogen (e.g. amines or guanidines). However other head-groups, e.g. arsonium and phosphonium have been reported [135]. Arsonium is less toxic than arsenic (III), and in vitro cytotoxicity evaluation showed that arsonium and phosphonium are surprisingly less toxic than the ammonium group [135, 136].

One property that can be changed by controlling the type of the head-group is the head-group cross-sectional area. The greater the difference between the cross-sectional area of the polar head-group and that of the hydrophobic domain, when the former is designed to be smaller than the latter, the greater is the ability of the cationic lipid to fuse with the cell membrane and endosomal membrane and the greater is the release of polynucleotides from their complex with the cationic lipid due to the decreased structural stability of the lipid assembly [133, 137]. The hydration of the head-group affects its cross-sectional area, thus, the conjugation of groups which decrease the hydration state (such as hydroxyalkyl groups that form intermolecular H-bonds) decreases the head-group cross-sectional area.

Thus, gene delivery by DOTMA and DOTAP (1,2-dioleoyloxy-3-(trimethylammonio)propane) was enhanced by incorporation of a hydroxyethyl group to yield the lipids DORIE

(1,2-dioleoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide) and DORI (*N*-[1-(2,3-dioleoyloxy)propyl-*N*-[1-(2-hydroxy)ethyl]-*N,N*-dimethyl ammonium iodide) respectively [138, 139]. The head-group cross-sectional area can be also controlled by subtle changes to the head-group structure. The DC-Chol (3 β -(*N,N'*-dimethylaminoethane)carbamoyl)cholesterol) with dimethylamino head-group resulted in more efficient transfection compared to DC-Chol with diethylamino or diisopropylamino head-groups, probably due to increased steric repulsion of the head-groups.

The *in vitro* gene transfer with six non-cholesterol-based cationic lipids (each having two alkyl chains) with a single guanidinium head-group in Chinese hamster ovary (CHO), COS-1, MCF-7, A549, and HepG2 cells has been reported [140]. These lipids were able to form lipoplexes with size-range 200-600 nm and ζ -potential +3.4 to -34 mV. The efficiencies of the lipids which had an extra quaternized cationic centre were 2-4-fold more than that of the commercially available reagent Lipofectamine in transfecting COS-1, CHO, A-549, and MCF-7 cells. MTT viability assay in CHO cells showed high (>75%) cell viabilities at the lipid/DNA charge ratios used. DNase I protection assays showed that the lipids having the extra quaternized centre protected DNA better against enzyme catalysed hydrolysis. These results shed light on the importance of choosing the type of head-group and number of cationic centres in designing cationic lipids [140].

A series of cationic cholesterol derivatives were synthesized by covalently attaching the heterocycles imidazole, piperazine, pyridine, and morpholine groups (the head-groups) to the parent cholesterol via a biodegradable carbamoyl linker [141]. These lipids were compared with the parent DC-Chol with the linear amine head-group, and they generally showed better or comparable transfection efficiency of pCMV-luciferase into human HepG2 cells (a human liver cancer cell line) in the presence or absence of FCS. The most efficient two of these lipids were with morpholine and piperazine head-groups, and they gave higher levels of gene expression in HepG2 and human melanoma cell line (K22) which are generally very hard to transfect with the commonly used reagents e.g. DC-Chol, Lipofectamine, and PEI. *In vivo* studies with lipids having morpholine and piperazine head-groups resulted in successful delivery of the reporter gene to the target cells through intrasplenic injection [141]. Cationic lipids which have more than one cationic head-group (multivalent cationic lipids) have more surface charge density than their monovalent (with one head-group) analogues, and they are generally expected to better bind and complex polynucleotides. Many multivalent cationic lipids contain a natural occurring polyamine such as spermidine and spermine, which are believed to interact with the minor groove of B-DNA [142].

The triamine spermidine and the tetramine spermine (Figure 6), and their diamine precursor putrescine, are organic polycations that are widely but unevenly distributed in both mammalian and non-mammalian cells and tissues. They have an essential role in controlling DNA, RNA and protein synthesis during normal and neoplastic growth, in cell differentiation, and tissue regeneration [143]. These polyamines exhibit many metabolic and neurophysiological effects in the nervous system, and are important for the developing and mature nervous system and affect modulation of ionic channels and calcium-dependent transmitter release [143-149].

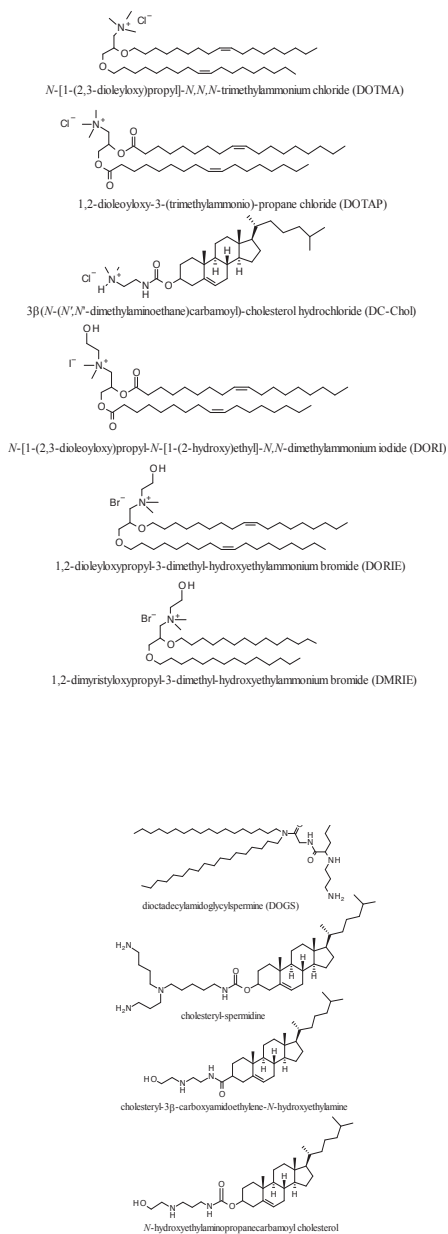


Figure 7. Representative examples of cationic lipids used in DNA and siRNA delivery.

Spermine is incorporated in the cationic polymer polyspermine imidazole-4,5-imine (PSI) and in dioctadecylamidoglycyl-spermine (DOGS) [150] (Figure 7); spermidine is bound in cholesteryl-spermidine [151]. The free amine groups of spermine in cholesteryl-spermine conjugates have different pK_a values and provide a buffering effect in the endosomes facilitating the escape of lipoplex from the endosomes [152]. The length of the linear polyamine attached to the hydrophobic domain and the charge distribution on it affects the transfection efficiency of the cationic lipid [153]. Addition of amine groups separated by methylene groups to the linear polyamine attached to a cholesterol residue did not automatically increase transfection efficiency regardless of the increased charge density. Molecular modelling simulations suggested that increasing chain length led to an increased number of folded conformations due to greater flexibility of the conjugates, which is unfavourable for interaction with DNA [132, 153].

The central tetramethylene motif of spermine was reported to be essential in conferring high transfection efficiency in a series of cholesterol-polyamine conjugates [152]. It was suggested that the tetramethylene segment of spermine can bridge between the DNA complementary strands, while the polyamine with a central trimethylene segment would only bind with the adjacent phosphates on the same DNA strand. These results point to the importance of the structure of the polyamine head-group and the relation between its amine groups, and also point to the fact that increasing efficiency of transfection is not only a matter of increasing the number of positive charges per head-group.

4.3. The hydrophobic domain

The length, saturation state and type of the hydrophobic chains conjugated to cationic lipids affect their transfection efficiency [154-156]. Although these factors were studied extensively for the effect on transfection, and although the majority of studies accepted that the type of alkyl chains influence the outcome of transfection, it is difficult to set a definitive set of rules to describe the best type of alkyl chains to be conjugated to the polar head-groups. The contribution of the alkyl chains (and the hydrophobic domain) to the cationic lipid properties as a whole is what determines the transfection efficiency of the lipid.

Results obtained with DMRIE (1,2-dimyristyloxypyl-3-dimethylhydroxyethyl-ammonium bromide) [157], glycine betaine conjugates [138] with two alkyl chains, alkyl acyl carnitine esters having chains of length C12 to C18 [158], lactic acid conjugates of *N,N*-dialkyl amine group [159], lipids related to DOTAP with two alkyl chains (C12-C18) linked to the head-group through ether bonds [160], and cationic lipids with different hydroxyethyl or dihydroxypropyl ammonium backbones and esterified hydrocarbon chains and hydroxyl substituents [161] showed that a comparison of the cationic lipids based only on the lengths of the two saturated aliphatic chains led to the observation of the superior transfection efficiency of C14 chains over the longer C16 and C18 chains [132, 133]. It was proposed that a shorter chain length facilitates mixing with cellular membranes [138] which is important for endosomal escape [162].

In another set of experiments, we showed the longer chain C18 oleoyl (with one *cis*-double bond) to be more efficient than cationic lipids with shorter chain lengths. Varying the chain length in *N*⁴,*N*⁹-diacyl spermines from C10 to C18, for plasmid DNA delivery, resulted in us establishing that the conjugate with C18 oleoyl chains is both more efficient and less toxic than

the shorter chain conjugates [163]. A series of multivalent Gemini-surfactants with the hydrophobic chains systematically varied resulted in the conjugates with C18 oleoyl chains to be better in transfection than the C16 and C14 alkyl chains [164]. Chain saturation was also shown to affect the efficiency of transfection. The results of studies on a set of cationic triester phosphatidyl choline derivatives (each having two alkyl chains) show a strong dependence of their transfection efficiency on the lipid hydrocarbon chain characteristics, where transfection activity increases with increasing chain unsaturation from fully saturated to having two double bonds. Transfection efficiency decreased with increasing chain length (increasing the total number of carbons per lipid molecule ~30-50). Maximum transfection was with monounsaturated myristoleoyl 14:1 chains [156]. The data obtained from transfection experiments with 20 cationic phosphatidylcholine (PC) derivatives show that hydrocarbon chain variations results in transfection efficiencies that varies by more than 2 orders of magnitude. The most important variables were chain saturation state and total number of carbon atoms in the lipid chains. Transfection increased with decreasing chain length and increasing chain unsaturation. Best transfection efficiency was found for cationic lipids with monounsaturated (myristoleoyl) 14:1 chains [154]. Higher levels of transfection were also reported with lipids having oleoyl chains in comparison with stearoyl chains [157, 158]. Unsaturated chains promote lipid fusion between the lipoplexes and the various cellular membranes, which is essential for delivery and endosomal escape [133, 154, 165].

Cholesterol derivatives with various cationic head-groups were synthesized to investigate their efficiency as siRNA delivery vectors. The transfection efficiencies of siRNA lipoplexes prepared with the cationic cholesterol derivatives DC-Chol, cholesteryl- β -carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol), and *N*-hydroxyethylaminopropane carbamoyl cholesterol (HAPC) was investigated in human prostate tumour cells that stably express the luciferase gene (PC-3-Luc). When lipoplexes were prepared in water, HAPC was more effective in knocking-down luciferase activity than OH-Chol and DC-Chol [166]. The presence of NaCl while preparing the lipoplexes increased the gene silencing efficiency of luciferase, while it did not affect efficiency of HAPC. The commercially available transfection reagent, Lipofectamine 2000 (a cationic lipid liposomal preparation) resulted in strong gene silencing by siRNA, but exhibited increased toxicity (~40% cell viability), in contrast to OH-Chol, DC-Chol, and HAPC lipoplexes (~80-100% cell viability). These results indicated that siRNA lipoplexes prepared with OH-Chol, and HAPC can efficiently suppress gene expression without increased cytotoxicity [166].

4.4. The linker

The linker is dependent upon the type (hence properties) of the functional group and its length (number of carbon atoms). The linker has two main functions: (a) to conjugate covalently the polar head-group to the hydrophobic domain; (b) to control the biodegradability of the cationic lipid and/or introduce a new property to the cationic lipid, e.g. responding to the intracellular reducing environment [133, 167]. The most commonly used linker functional groups are: amide, carbamate, ester, ether, ortho ester, and disulfide.

Both amides and ester bonds are biodegradable and hence are hypothesized to be less toxic than other non-biodegradable bonds (e.g. ethers) [168]. Lipids with a pyridinium head-group (with palmitoyl 16:0 hydrophobic domains and with ester and amide linkers) were used to prepare liposomes with either DOPE or cholesterol at the cationic lipid/helper-lipid molar ratio of 1:1. Following transfection of CHO cells with lipoplexes delivering plasmids expressing EGFP, the cationic lipids having amide linkers significantly increased transfection efficiency in all liposomal formulations compared to their counterparts having the ester linker [169]. The high transfection efficiency of lipids with amide linker was suggested to be due to their lower phase-transition temperature which makes the liposome's bilayer structure more stable in aqueous media during the transfection process as well as liposome storage. The phase-transition temperature of a lipid is the temperature at which there is a change in the lipid's physical state from the ordered gel phase (where the hydrocarbon chains are closely packed and fully extended) to the disordered liquid crystalline phase (where the hydrocarbon chains are fluid and randomly orientated) [169].

Depending on the structure of the cationic lipid, the linker influence on transfection efficiency can be more than on cytotoxicity. Cholesterol-based cationic lipids that have different nitrogenous heterocyclic head-groups (*N*-methylimidazole, *N*-methylmorpholine, and pyridine) and acid-labile linkers (carbamate, ester, and *N,O*-acetal ether) were used to transfect human embryonic kidney 293 (HEK 293) cells with EGFP plasmid [170]. Choosing those linkers was based on the concept that incorporation of acid-labile bonds in the cationic lipid structure enhances the release of polynucleotides from the endosomes, therefore increasing the transfection efficiency [171]. *N,O*-Acetals are known to undergo hydrolysis in acidic environment [170, 171]. The results showed that the structure of these lipids only slightly affected their cytotoxicity but largely changes the efficiency of intracellular accumulation of the polynucleotides. The lipids having the cationic head-groups pyridine and/or methylimidazole head-groups with either an ester or a carbamate linker resulted in better transfection efficiency as compared with the cationic lipids with either the *N*-methylmorpholine head-groups and/or an ether linker. The lipid that has a pyridine head-group and a carbamate linker to deliver EGFP plasmid resulted in comparable transfection efficiency with that achieved with commercially available Lipofectamine 2000.

Two cleavable cationic lipids having a linear or a cyclic ortho-ester linker between the cationic head-group and the unsaturated hydrophobic domain (two oleoyl chains) were previously reported [172]. It is hypothesized that the acidic pH in the endosomes catalyzes the hydrolysis of the linker group to result in fragmentation products that destabilize the endosomal membranes. At pH 7.4, the lipids (with DOPE) formed stable lipoplexes with plasmid DNA. Decreasing the pH enhanced the hydrolysis of the ortho ester linkers which removed the cationic head-groups and caused lipoplex aggregation. At pH 5.5, the cationic lipid *N*-[2-methyl-2-(1',2'-dioleylglyceroxy)dioxolan-4-yl]methyl-*N,N,N*-trimethylammonium iodide that have a cyclic ortho-ester linker showed increased pH-sensitivity and caused the permeation of its lipoplexes to model biomembranes within the time span of endosomal processing before the lysosomal degradation. This lipid markedly increased gene transfection (~3-50-fold) of the luciferase reporter protein in monkey kidney fibroblast (CV-1) and human breast cancer (HTB-129) cells in culture compared to the pH-insensitive control lipid DOTAP lipoplexes [172].

Transfection with DNA lipoplexes of three thiocholesterol-derived gemini cationic lipids possessing disulfide linkages incorporated between the cationic head-group and the thiocholesterol backbone in order to render the lipids biodegradable has been reported [173]. Comparing transfection in a prostate cancer line (PC3AR) and a human keratinocyte cell line (HaCat) with two commercially available reagents showed comparable or better expression of GFP in the transfected cells. Cytotoxicity studies showed the nontoxic property of these lipid-DNA complexes at different *N/P* ratios used for transfection studies. The rationale behind this design was to ensure the destabilization of the lipid-polynucleotide lipoplexes in the cytoplasm after reduction of the disulfide linker by the intracellular glutathione (GSH), which is the most abundant low molecular weight thiol present in cells and is involved in controlling cellular redox environment. GSH is found at very high intracellular concentrations and at comparatively low extracellular concentrations e.g. blood plasma concentrations (2 μ M) are 1000-fold less than concentration in erythrocytes (2 mM). This large difference between intra- and extracellular environments provides a potential mechanism for release of polynucleotides from lipoplexes of lipids that have a disulfide functional group linker and is now a well-trodden research path [115, 134, 173].

5. Conclusions and future avenues

In our research, symmetrical and asymmetrical acyl polyamine derivatives (fatty acid amides of spermine) [152] have been synthesized, characterized, and evaluated as non-viral vectors for siRNA [163, 174-177]. The intracellular delivery of siRNA and the subsequent sequence specific gene silencing has been quantified by flow cytometry techniques (FACS analysis) [163]. The ability of the spermine conjugates to bind siRNA and form nanoparticles has been investigated and the effect of the complexes of siRNA lipoplexes on the cell viability 48 h post-transfection has been quantified. Our SAR studies allow the identification of the most efficient fatty acids in terms of high gene-silencing efficiency and high cell viability [174-178].

Whilst we were completing this Chapter, four interesting papers, each one on a different aspect of this topic, were published. Langer, Anderson and co-workers at MIT reported on the delivery of immunostimulatory RNA (isRNA) to Toll-like receptor (TLR)-expressing cells to drive innate and adaptive immune responses. The specific activation of TLRs has potential for a variety of therapeutic indications including antiviral immunotherapy and as vaccine adjuvants. Effective lipidoid-isRNA nanoparticles, when tested in mice, stimulated strong IFN- α responses following subcutaneous injection, had robust antiviral activity that suppressed influenza virus replication, and enhanced antiovalbumin humoral and cell-mediated responses when used as a vaccine adjuvant. Their lipidoid formulations, designed specifically for the delivery of isRNA to TLRs, were superior to the commonly used *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate-RNA delivery system and may provide new tools for the manipulation of TLR responses *in vitro* and *in vivo* [179]. This paper follows after their other recent major contribution on delivering naked siRNA as part of a self-assembled (due to DNA complementarity) tetrahedral nanoparticle construct considering the presentation of folate as a cancer targeting ligand [180]. These monodisperse nanoparticles of

essentially naked DNA, carrying siRNA as the cargo, have a defined size of only a few nm. They show that at least three folate molecules per nanoparticle are required for optimal delivery of the siRNA into cells and that gene silencing only occurs when the ligands are appropriately orientated. In vivo, these naked DNA nanoparticles showed a longer blood circulation time than the parent siRNA [180]. In another exciting development, Geall and co-workers at Novartis have also advanced the field of nucleic acid vaccines by taking advantage of the recent innovations in non-viral systemic delivery of siRNA using lipid nanoparticles (LNPs) to develop a self-amplifying RNA vaccine. This technology elicited broad, potent, and protective immune responses, comparable with those achieved by a viral delivery system, but without the inherent limitations of viral vectors [181]. Even today, a biologically responsive cationic polymer system based on spermine has been reported for the intracellular delivery of siRNA [182]. This polyspermine imidazole-4,5-imine (PSI) (Figure 7) carrier is designed to be hydrolysed at the mildly acidic pH found in the endosome.

It is clear that both ssRNA to activate the immune system and RNAi brought about by siRNA delivery have high therapeutic potential. The major remaining barrier, that of efficient and potentially selective delivery to target cells is now being addressed. The non-viral delivery of siRNA is a major tool in modern functional genomics. Medicines design, the formulation of drugs, in this case siRNA and plasmid DNA, is an essential requirement for efficient gene therapy.

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Gene Therapy Tools: Biological

Mesenchymal Stem Cells as Gene Delivery Vehicles

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Additional information is available at the end of the chapter

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1. Introduction

Mesenchymal stem cells (MSCs) possess a battery of unique properties which make them ideally suited not only for cellular therapies/regenerative medicine, but also as vehicles for gene delivery in a wide array of clinical settings. These include: 1) widespread distribution throughout the body; 2) ease of isolation and ability to be extensively expanded in culture without loss of potential; 3) the ability to differentiate into a wide array of functional cell types in vitro and in vivo; 4) they exert pronounced anti-inflammatory and immunomodulatory effects upon transplantation; and 5) the ability to home to damaged tissues, solid tumors, and metastases following in vivo administration.

In this Chapter, we will summarize the latest research in the use of MSC in regenerative medicine, focusing predominantly on their use as vehicles for transferring exogenous genes. To highlight the immense potential these cells possess for gene therapy applications, we will attempt to paint as broad a canvas as possible, starting with a discussion about the basic biology of MSC, and their unique properties which combine to make MSC one of the most promising stem cell populations for use in gene therapy studies and trials. We will reveal the versatility of MSC as gene delivery vehicles by summarizing some of the most recent studies showing the ease with which MSC can be modified with a wide range of both viral and non-viral vector systems, and highlighting some of the advantages to delivering transgenes within a cellular vehicle, as opposed to administering vectors directly into the body. We will then discuss the engineering of MSC to enhance their natural abilities to mediate repair within various tissues; one of the most popular uses of MSC to-date in the gene therapy arena. We will discuss our recent work, and that of others, using MSC to deliver coagulation factors to treat the hemophilias, with hemophilia A serving as a paradigm for how MSC could be used to deliver a therapeutic transgene, and thereby correct essentially any inherited disease. The Chapter will conclude with a discussion of MSC's ability to selectively migrate to forming solid tumors following intravenous administration, and to actively seek out

metastases at sites far removed from the primary site of the tumor. We will summarize exciting recent work showing that it is possible to exploit this property to achieve sustained, high-level expression of pro-apoptotic gene products within the tumor, obtaining greatly improved anti-tumor effects, while essentially eliminating the systemic toxicities that plague current radiation/chemotherapy-based treatments.

2. Isolation and characterization of MSC

More than 30 years ago, Friedenstein pioneered the concept that the marrow microenvironment resided within the so-called stromal cells of the marrow, by demonstrating that fibroblastoid cells obtained from the bone marrow were capable of transferring the hematopoietic microenvironment to ectopic sites [1, 2]. Years later, scientists began to explore the full potential of these microenvironmental cells, and results of these studies led to the realization that this population harbored cells with properties of true stem cells. These cells were officially termed mesenchymal stem cells (MSC) [3]. MSC are now recognized to be a key part of the microenvironment/niche that supports the hematopoietic stem cell and drives the process of hematopoiesis, yet despite serving this vital function, MSC only comprise ~0.001-0.01% of cells within the marrow [4], making methods for isolating/enriching and for expanding these cells essential to their study and their ultimate clinical use. The most straightforward method for obtaining MSC is to exploit their propensity to adhere to plastic and their ability to be passaged with trypsin (contaminating hematopoietic cells do not passage with trypsin) to rapidly obtain a relatively morphologically homogeneous population of fibroblastic cells from a bulk mononuclear cell preparation [5-7]. Unfortunately, true MSC (defined by function) account for only a small percentage of the highly heterogeneous resultant population, making results obtained with cells prepared in this fashion difficult to interpret, and inconsistent from experiment-to-experiment and from group-to-group. The identification of antigens that are unique to MSC would eliminate this problem. Human MSC do not express markers which have been associated with other stem cell populations (like hematopoietic stem cells) such as CD34, CD133, or c-kit, nor hematopoietic markers such as CD45, CD14, and CD19. Moreover, no marker has been identified to date that specifically identifies MSC. Nevertheless, several surface antigens have proven useful for obtaining highly enriched MSC populations. The first of these markers to be identified was Stro-1, an antibody that reacts with non-hematopoietic bone marrow stromal precursor cells [8]. Although the antigen recognized by this antibody has not yet been identified, we and others have found that by tri-labeling bone marrow cells with Stro-1, anti-CD45, and anti-GlyA, and selecting the Stro-1+CD45-GlyA- cells, it is possible to consistently obtain a homogeneous population that is highly enriched for MSC [9-15]. In addition to Stro-1, antibodies such as SB-10, SH2, SH3, and SH4 have been developed over the years and numerous surface antigens such as CD13, CD29, CD44, CD63, CD73, CD90, CD105, and CD166 have been used to attempt to identify and isolate MSC [16-18]. Unfortunately, all of these antigens appear to be expressed on a wide range of cell types within the body in addition to MSC. This lack of a unique marker suggests that to obtain a pure population of MSC that are functionally homo-

geneous, investigators will likely either have to await the development of novel antibodies that recognize as yet unidentified antigens that are unique to primitive MSC, or employ strategies in which multiple antibodies are combined to allow for positive selection of MSC and depletion of cells of other lineages that share expression of the antigens recognized by the MSC antibody in question, as we have done with Stro-1, CD45, and GlyA.

3. Sources of MSC

Although much of the work to date has focused on MSC isolated from adult bone marrow, we and others have isolated cells that appear phenotypically and functionally to be MSC, from numerous tissues including brain, liver, lung, fetal blood, umbilical cord blood, kidney, and even liposuction material [19-26]. The broad distribution of MSC throughout the body leads one to postulate that MSC may play a critical role in organ homeostasis by providing supportive factors and/or mediating maintenance/repair within their respective tissue. Importantly, although MSC from each of these tissues appear similar with respect to phenotype and overall differentiative potential, studies at the RNA and protein level have now revealed that subtle differences exist between MSC from these various tissues, with MSC from each tissue possessing a molecular fingerprint indicative of their tissue of origin [21, 22, 27-31]. Using a non-injury fetal sheep transplantation model, we showed that these differences result in a bias for human MSC to home to and give rise to cells of their tissue of origin *in vivo* [32, 33]. This suggests that, to use MSC as therapeutics or as gene delivery vehicles, the ideal source of MSC may differ depending on the specific disease to be treated and the desired target organ.

Despite the apparent presence of MSC within many of the major organs of the body, the relatively non-invasive fashion with which adipose tissue or bone marrow can be obtained, and the fact that both these tissues could readily be obtained autologously, combine to suggest that these two tissues will likely be the predominant source of MSC employed in clinical applications in the foreseeable future. However, additional experiments will need to be performed to rigorously assess the inherent safety of adipose tissue-derived MSC before these cells will see widespread clinical use, since several recent studies have suggested that they may be inherently less genetically stable than MSC isolated from bone marrow [34], exhibiting aneuploidy [35, 36] and undergoing transformation [37, 38] upon prolonged propagation *in vitro*. However, another recent study provided evidence that, even if genomic instability is intentionally induced with genotoxic agents, adipose tissue-derived MSC respond to this insult by undergoing terminal adipogenic differentiation rather than transformation [39]. The dramatically conflicting nature of the results from these different studies could, perhaps, be due to differing methods employed for isolating and culturing MSC, differing levels of contaminating non-MSC cells in the cultures, as well as the duration of the culture (i.e., the number of times the cells have been passaged). Bearing this possible instability in mind, the recommendation has been put forward to only make clinical use of cells that have been passaged fewer than 25 times in culture, regardless of the source of MSC [40].

4. MSC as vehicles for delivering therapeutic genes

While MSC possess tremendous therapeutic potential by virtue of their ability to lodge/engraft within multiple tissues in the body and both give rise to tissue-specific cells and release trophic factors that trigger the tissue's own endogenous repair pathways [41-59], gene therapists have realized that these properties are just the beginning of the therapeutic applications for MSC [24, 60, 61]. By using gene therapy to engineer MSC to either augment their own natural production of specific desired proteins or to enable them to express proteins outside of their native repertoire, it is possible to greatly broaden the spectrum of diseases for which MSC could provide therapeutic benefit. Unlike hematopoietic stem cells which are notoriously difficult to modify with most viral vectors while preserving their *in vivo* potential, MSC can be readily transduced with all of the major clinically prevalent viral vector systems including those based upon adenovirus [62-64], the murine retroviruses [64-68], lentiviruses [69-74], and AAV [75, 76], and efficiently produce a wide range of cytoplasmic, membrane-bound, and secreted protein products. This ease of transduction coupled with the ability to subsequently select and expand only the gene-modified cells *in vitro* to generate adequate numbers for transplantation combine to make MSC one of the most promising stem cell populations for use in gene therapy studies and trials.

To date, the majority of studies using gene-modified MSC have been undertaken with the purpose of enhancing the natural abilities of MSC to mediate repair within various tissues. Using the heart as an example, once investigators discovered the identity of some of the key trophic factors responsible for MSC's beneficial effect on the injured myocardium, they undertook studies using MSC engineered to overexpress a number of these factors [69, 77-86]. As anticipated, the "gene-enhanced" MSC were substantially more effective than their unmodified counterparts, producing greatly enhanced therapeutic effects. Similar studies have also been performed to repair the damaged/diseased CNS using MSC engineered to produce neurotrophic factors [87-94], to repair the injured liver using MSC expressing proteins involved in hepatocyte differentiation and/or proliferation [95, 96], to repair ischemia/reperfusion injury [97-102], and to repair the kidney [103-105]. In each case thus far, MSC engineered to express higher levels of proteins known to be beneficial for the tissue in question and/or to promote survival have produced markedly better results than unmodified MSC.

Despite the many advantages of using MSC as gene delivery vehicles, however, relatively few studies have thus far explored this potential for the treatment of genetic diseases. One disease for which we and others are actively investigating MSC for delivery of a therapeutic gene is hemophilia A [106-112].

5. Hemophilia A as a paradigm for the use of gene-modified msc to correct genetic diseases

Hemophilia A represents the most common inheritable deficiency of the coagulation proteins [113]. The severity of hemophilia A is traditionally based on plasma levels of FVIII,

with persons exhibiting less than 1% normal factor (<0.01 IU/mL) being considered to have severe hemophilia, persons with 1-5% normal factor moderately severe, and persons with 5%-40% of the normal FVIII levels mild [114-116]. Up to 70% of hemophilia A patients present with the severe form of the disease, and suffer from frequent hemorrhaging, leading to chronic debilitating arthropathy, hematomas of subcutaneous connective tissue/muscle, and internal bleeding. Over time, the collective complications of recurrent hemorrhaging result in chronic pain, absences from school and work, and permanent disability [114]. Current state-of-the-art treatment consists of frequent prophylactic infusions of plasma-derived or recombinant FVIII protein to maintain hemostasis, and has greatly increased life expectancy and quality of life for many hemophilia A patients.

This treatment approach is, however, far from ideal, due to the need for lifelong intravenous infusions and the high treatment cost. Moreover, this treatment is unavailable to a large percentage of the world's hemophiliacs, placing these patients at great risk of severe, permanent disabilities and life-threatening bleeds. Furthermore, even among the patients who are fortunate enough to have access to, and the financial means to afford, prophylactic FVIII infusions, approximately 30% will form FVIII inhibitors [117]. The formation of these inhibitors greatly reduces the efficacy of subsequent FVIII infusions, and can ultimately lead to treatment failure, placing the patient at risk of life-threatening hemorrhage. There is thus a significant need to develop novel, longer-lasting hemophilia A therapies.

In the past three decades, the remarkable progress in the understanding of the molecular basis of the disease, the identification and characterization of FVIII gene, structure, and biology has heightened the interest and feasibility of treating hemophilia A with gene therapy. In contrast to current protein-based therapeutics, lifelong improvement or permanent cure of hemophilia A is theoretically possible after only a single gene therapy treatment; indeed, several aspects of hemophilia A make it ideally suited for correction by gene therapy [118-126]. First, in contrast to many other genetic diseases, the missing protein (coagulation FVIII) does not need to be expressed in either a cell- or tissue-specific fashion to mediate correction. Although the liver is thought to be the primary natural site of synthesis of FVIII, expression of this factor in other tissues exerts no deleterious effects. As long as the protein is expressed in cells which have ready access to the circulation, it can be secreted into the bloodstream and exert its appropriate clotting activity. Second, even modest levels (3-5%) of FVIII-expressing cells would be expected to convert severe hemophilia A to a moderate/mild phenotype, reducing or eliminating episodes of spontaneous bleeding and greatly improving quality of life. Thus, even with the low levels of transduction that are routinely obtained with many of the current viral-based gene delivery systems, a marked clinical improvement would be anticipated in patients with hemophilia A. Conversely, even supra-physiologic levels of FVIII as high as 150% of normal are predicted to be well tolerated, making the therapeutic window extremely wide [116]. Based on this knowledge, the American Society of Gene and Cell Therapy (www.ASGCT.org) recently provided NIH director, Dr. Francis Collins, with a roadmap of disease indications that it feels will be viable gene therapy products within the next 5-7 years. The hemophilias were identified as belonging to the most promising, "Target 10", group of diseases.

6. Mesenchymal Stem Cells (MSC) as hemophilia A therapeutics

As discussed in the preceding section, the liver is thought to be the primary site of FVIII synthesis within the body. We and others have devoted a great deal of energy to demonstrating the ability of MSC from various sources to serve as therapeutics for liver disease [11, 13, 14, 33, 96, 127-152]. It is now clear that, not only do MSC have the ability to generate, *in vitro* and *in vivo*, cells which are indistinguishable from native hepatocytes, but transplantation of MSC in a range of model systems can result in fairly robust formation of hepatocytes which repair a variety of inborn genetic defects, toxin-induced injuries, and even fibrosis. The fetal sheep model provides a unique system in which to explore the full differentiative potential of various stem cell populations, since the continuous need for new cells within all of the organs during fetal development provides a permissive milieu in which gene-modified donor cells can engraft, proliferate, and differentiate. Furthermore, by performing the transplant at a stage in gestation when the fetus is considered to be largely immuno-naïve, it is possible to engraft human cells at significant levels, which persist for the lifespan of the animal due to induction of donor-specific tolerance [130-132]. Indeed, in ongoing studies, we have found that, after transplantation into fetal sheep, human MSC engraft at levels of up to 12% within the recipient liver [11, 131, 132, 153-158], and contribute to both the parenchyma and the perivascular zones of the engrafted organs, placing them ideally for delivering FVIII into the circulation. Since FVIII levels of 3-5% of normal would convert a patient with severe hemophilia A to a moderate or mild phenotype, these levels of engraftment should be highly therapeutic. These collective results thus suggest that MSC may represent an ideal cell type for treating hemophilia A.

However, although MSC engrafted (following transplantation *in utero*) at significant levels within organs that are natural sites of FVIII synthesis, only a small percent expressed endogenous FVIII, suggesting that simply transplanting "healthy" MSC will not likely provide an effective means of treating/curing hemophilia A. By using gene therapy to engineer MSC to express FVIII, however, it is highly probable that the levels of engrafted MSC we have thus far achieved *in utero* would provide marked therapeutic benefit in hemophilia A. By transducing the MSC *in vitro*, rather than performing gene therapy by injecting the vector directly, as is the current practice in clinical gene therapy trials, there is no risk of off-target transduction, and the vector being employed simply needs a strong constitutively active promoter to ensure that all cells derived from the transplanted MSC continue to express FVIII and mediate a therapeutic effect. Importantly, the only documented cases of retroviral-induced insertional mutagenesis have been observed following genetic modification of hematopoietic stem cells [159-161]; there is no evidence that MSC transform or progress to clonal dominance following transduction, suggesting they represent safe cellular vehicles for delivering FVIII (or other transgenes).

Importantly, critical proof-of-principle studies have already shown that MSC can be transduced with FVIII-expressing viral vectors and secrete high levels of FVIII protein *in vitro* and following transplantation *in vivo* [106-109]. FVIII purified from the conditioned medium of the transduced MSC was proven to have a specific activity, relative electrophoretic

mobility, and proteolytic activation pattern that was virtually identical to that of FVIII produced by other commercial cell lines [109]. Given the widespread distribution and engraftment of MSC following their systemic infusion, the ability of MSC to give rise, *in vivo*, to cells of numerous tissue types, and their ability to efficiently process and secrete high amounts of biologically active FVIII, they are, not surprisingly, being viewed as ideal vehicles for delivering a FVIII transgene throughout the body and thus providing long-term/permanent correction of hemophilia A [106-109, 162].

In addition to their widespread engraftment and their ability to serve as delivery vehicles for the FVIII gene, the rather unique immunological properties of MSC may further increase their utility for treating hemophilia A. MSC do not normally express MHC class II or the costimulatory molecules CD80 and CD82, unless they are stimulated with IFN- γ , and are thus viewed as being relatively hypo-immunogenic. As such, they do not provoke the proliferation of allogeneic lymphocytes or serve as very effective targets for lysis by cytotoxic T cells or NK cells. In fact, a large body of evidence is now accumulating that MSC can be readily transplanted across allogeneic barriers without eliciting an immune response [163, 164]. Thus, if one wished to use MSC to treat hemophilia A, off-the-shelf MSC from an unrelated donor could theoretically be used, greatly increasing the feasibility of obtaining and using these stem cells for therapy.

Perhaps even more important from the standpoint of their potential use as hemophilia A therapeutics, more recent studies have provided evidence that MSC also appear to have the ability to exert both immunosuppressive and anti-inflammatory properties both *in vitro* and *in vivo*. These properties appear to result from MSC's ability to intervene, at multiple levels, with the generation and propagation of an immune response. To name just a few examples, MSC have been demonstrated to interfere with the generation and maturation of cytotoxic and helper T cells [165-174], dendritic cells [175-178], and B cells [179]. In addition to actively shutting down the generation of immune effector cells, MSC also have the ability to induce the formation of potent Tregs, although the mechanism by which this comes about is still the subject of active research [40, 180-182]. MSC are also known to express a battery of factors [40, 168-170, 180, 183-187] that reduce local inflammation, blunt immune response, and counteract the chemotactic signals responsible for recruiting immune cells to sites of injury/inflammation. One could thus envision these immune-dampening properties enabling the delivery of FVIII without eliciting an immune response and subsequent inhibitor formation, thus overcoming one of the major hurdles that plague current treatment/management of hemophilia A. As will be discussed in the next section, however, our postnatal studies in the hemophilic sheep suggest that further work will be required to discover how to obtain these potential immune benefits in the context of the ongoing injury/inflammation present in animals/patients with clinically advanced hemophilia A.

In addition to the aforementioned properties, preclinical animal studies examining the potential of MSC isolated from adult tissues have also highlighted another interesting and clinically valuable characteristic of MSC; their ability to selectively navigate to sites of injury and/or inflammation within the body. Once reaching these specific sites, the MSC then mediate repair both by engrafting and generating tissue-specific cells within the injured tissue

[188-190], and by releasing trophic factors that blunt the inflammatory response and often promote healing by activating the tissue's own endogenous repair mechanisms. While the mechanisms responsible for this trafficking to sites of injury are still being elucidated [191-193], this observation raises the exciting possibility that, following systemic infusion, FVIII-expressing MSC could efficiently migrate to sites of active bleeding/injury, thereby releasing FVIII locally and focusing the therapy where it is most needed. As will be discussed in the following section, over the past 2-3 years, we have begun exploring whether it is possible to exploit these many advantages of MSC as a cellular vehicle for delivering a FVIII gene by testing the ability of FVIII-expressing MSC to correct hemophilia A in a new large animal model; sheep.

7. Establishment of a new preclinical model of hemophilia A and success with MSC-based treatment

A number of animal models have been developed to evaluate new methods of not only treatment of coagulation disorders, but also the prevention and treatment of inhibitor formation. Transient hemophilic rabbit models induced by infusion of plasma containing inhibitors have been used to evaluate the effect of different bypass products to factor VIII [194], but these models, while valuable for inhibitor studies, do not accurately recapitulate the human disease, precluding their use for gene therapy studies. Fortunately, dog models of hemophilia A with congenital deficiency [195, 196] and mouse models obtained by gene targeting and knockout technology [197] are available to study FVIII function and gene therapy approaches for treating hemophilia A. Therapeutic benefit has been obtained in numerous studies using a variety of vector systems in the murine model [121, 122, 198-204], and phenotypic correction of hemophilia A in the dog has been achieved, but has proven to be far more difficult than in mice [205, 206]. Despite promising results in both canine and murine models, however, no clinical gene therapy trial has shown phenotypic/clinical improvement of hemophilia A in human patients. This is in marked contrast to the recent clinical successes with gene therapy for hemophilia B [207]. The reasons for the disparity in the efficacy of gene therapy for treating hemophilia A versus B is not presently clear. Nonetheless, based on the disappointing results to-date, there are currently no active hemophilia A clinical gene therapy trials, even though hemophilia A accounts for roughly 80% of all cases of hemophilia.

The difficulties seen thus far translating success in animal models into therapeutic benefit in human patients underscores the importance of preclinical animal models that both precisely mimic the disease process of hemophilia A, and closely parallel normal human immunology and physiology. To this end, between 1979 and 1982, a number of male offspring of a single white alpine ewe at the Swiss Federal Institute of Technology all died several hours postpartum due to severe bleeding from the umbilical cord [208-210]. Daughters and granddaughters of this ewe also gave birth to lambs exhibiting the same pathology. Investigation of the affected animals showed extensive subcutaneous and intramuscular hematomas. Spontaneous hemarthroses were also frequent, leading to reduced locomotion and symp-

toms of pain in standing up, restricting nursing activity. Stronger injuries that arose when animals were not placed in carefully controlled isolation resulted in heavy bleeding and intensive pain. Laboratory tests showed increased PTT, and FVIII levels (as assessed by aPTT) of about 1% of control animals. Replacement therapy with human FVIII (hFVIII) concentrate or fresh sheep plasma resulted in remission of disease and rapid clinical improvement.

Unfortunately, due to the expense and effort of maintaining these sheep, the Swiss investigators allowed the line to die out, saving only a few straws of semen prior to allowing this valuable resource to pass into extinction. We recently used a variety of reproductive technologies to successfully re-establish this line of hemophilia A sheep, and fully characterized both the clinical parameters and the precise molecular basis for their disease [211-216]. In similarity to mutations seen in many human patients [217], these animals possess a premature stop codon with a frameshift mutation. This is the only animal model of hemophilia A with this clinically relevant mutation-type, providing a unique opportunity to study therapies in this context. All ten animals to-date have exhibited bleeding from the umbilical cord, prolonged tail and "cuticle" bleeding time, and multiple episodes of severe spontaneous bleeding including hemarthroses, muscle hematomas, and hematuria, all of which have responded to human FVIII. Just like human patients with severe hemophilia A, a hallmark symptom in these sheep is repeated spontaneous joint bleeds, which lead to chronic, debilitating arthropathies and reduced mobility. Importantly, chromogenic assays performed independently at the BloodCenter of Wisconsin and Emory University revealed undetectable FVIII activity in the circulation of these sheep, explaining their severe, life-threatening phenotype.

In addition to the value of another large animal model of hemophilia A and the uniqueness of the mutation, sheep possess many characteristics that make them an ideal preclinical model for gene therapy. The first of these is the size. Sheep are fairly close in size to humans, weighing roughly 8lbs at birth and 150-200lbs as adults, likely obviating the need for scale-up of cell/vector dose to move from experiments in sheep to trials in humans. This is in marked contrast to mice which are ~2800 times smaller than a typical human patient [218]. Secondly, sheep share many important physiological and developmental characteristics with humans; for example, the pattern of fetal to adult hemoglobin switching, and the naturally occurring changes in the primary sites of hematopoiesis from yolk sac to fetal liver and finally to the bone marrow near the end of gestation. Thirdly, sheep are outbred, and thus represent a wide spectrum of genetic determinants of the immune response, as do humans. As the immune response to both the vector and the vector-encoded FVIII are likely to play a key role in FVIII inhibitor formation (or lack thereof), this represents an advantage not found in most other models, with the possible exception of the dog, which could conceivably be outbred as well to achieve a broader genetic spectrum. In addition, the development of the sheep immune system has been investigated in detail [219-225], making sheep well suited for studying the immunological aspects of gene-based therapies for hemophilia A. Importantly, the large size of the sheep, their long life span (9-12 years), and their relative ease of maintenance and breeding make it possible to conduct long-term studies in relatively large numbers of animals to fully evaluate the efficacy and safety issues related to gene

therapy. For these reasons, we feel that the sheep are a particularly relevant model in which to examine gene and cell-based therapies for hemophilia A. An additional unique advantage to using sheep to study hemophilia A treatment is that in sheep, like human, a large percentage of the vWF is found within platelets rather than free in plasma. This is in contrast to dog (in which vWF circulates free in plasma [226, 227]), and makes the sheep an ideal large animal model in which to explore the use of platelet-targeted gene therapy for hemophilia A [126, 228-230].

To experimentally test the ability of MSC to serve as FVIII delivery vehicles and thus treat hemophilia A, we recently tested a novel, non-ablative transplant-based gene therapy in 2 pediatric hemophilia A lambs [110-112]. During the first 3-5 months of life, both these animals had received frequent, on-demand infusions of human FVIII for multiple hematomas and chronic, progressive, debilitating hemarthroses of the leg joints which had resulted in severe defects in posture and gait, rendering them nearly immobile. In an ideal situation, one would use autologous cells to deliver a FVIII transgene, and thus avoid any complications due to MHC-mismatching. Unfortunately, the severe life-threatening phenotype of the hemophilia A sheep prevented us from collecting bone marrow aspirates to isolate autologous cells. We therefore elected to utilize cells from the ram that had sired the two hemophiliac lambs, hoping that, by using paternal (haploidentical) MSC, immunologic incompatibility between the donor and recipient should be minimized sufficiently to allow engraftment, especially given the large body of evidence now accumulating that MSC can be transplanted across allogeneic barriers without eliciting an immune response [163, 164].

Based on our prior work in the fetal sheep model, we knew that the intraperitoneal (IP) transplantation of MSC results in widespread engraftment throughout all of the major organs [11, 131, 157, 231-233] and durable expression of vector-encoded genes [232-234]. We further reasoned that using the IP route would also have the advantage of enabling the cells to enter the circulation in an almost time-release fashion, after being engulfed by the omentum and absorbed through the peritoneal lymphatics. Importantly, we also felt that the use of the IP route would enable us to avoid the lung-trapping which hinders the efficient trafficking of MSC to desired target organs following IV administration, and also poses clinical risks due to emboli formation [235, 236].

Following isolation, MSC were simultaneously transduced with 2 HIV-based lentivectors, the first of which encoded an expression/secretion optimized porcine FVIII (pFVIII) transgene [112]. We selected a pFVIII transgene for two reasons. First, we had not yet cloned the ovine FVIII cDNA and constructed a B domain-deleted cassette that would fit in a lentivector. Secondly, the pFVIII transgene had previously been shown, in human cells, to be expressed/secreted at 10-100 times higher levels than human FVIII [120, 121, 237]. We thus felt that these very high levels of expression/secretion might enable us to achieve a therapeutic benefit, even in the event we obtained very low levels of engraftment of the transduced paternal MSC. The second lentivector encoded eGFP to facilitate tracking and identification of donor cells *in vivo*. Combining the 2 vectors unexpectedly resulted in preferential transduction with the eGFP vector, such that only about 15% of the MSC were transduced with the pFVIII-encoding vector, as assessed by qPCR. Once the transduced MSC had been suffi-

ciently expanded, the first animal to be transplanted was treated with a dose of hFVIII calculated to correct the levels to 200%, to ensure no procedure-related bleeding occurred. The animal was then sedated, and 30×10^6 transduced MSC were transplanted into the peritoneal cavity under ultrasound guidance in the absence of any preconditioning.

Following transplantation, FVIII activity (assessed by chromogenic assay) was undetectable in the circulation, but this animal's clinical picture improved dramatically. All spontaneous bleeding events ceased, and he enjoyed an event-free clinical course, devoid of spontaneous bleeds, enabling us to cease hFVIII infusions. Existing hemarthroses resolved, the animal's joints recovered fully and resumed normal appearance, and he regained normal posture and gait, resuming a normal activity level. To our knowledge, this represents the first report of phenotypic correction of severe hemophilia A in a large animal model following transplantation of cells engineered to produce FVIII, and the first time that reversal of chronic debilitating hemarthroses has been achieved.

Based on the remarkable clinical improvement we had achieved in this first animal, we transplanted a second animal with 120×10^6 paternal MSC, >95% of which were transduced and expressing pFVIII. We anticipated that by transplanting 4x's the number of cells with roughly 6x's the transduction efficiency, we would achieve pronounced improvement and therapeutic levels of FVIII in the circulation of this animal. In similarity to the first animal, hemarthroses present in this second animal at the time of transplant resolved, and he resumed normal activity shortly after transplantation. This second animal also became factor-independent following the transplant. These results thus confirm the ability of this MSC-based approach to provide phenotypic correction in this large animal model of hemophilia A. However, just as we had observed in the first animal, no FVIII was detectable in the circulation of this animal, making the mechanism by which this procedure mediated such pronounced clinical improvement uncertain.

Despite the pronounced clinical improvement we observed in the first animal, he mounted a rapid and fairly robust immune response to FVIII, in similarity to prior studies performed with hemophilia A mice [237]. Before transplant, this first animal had Bethesda titers against hFVIII of only ~3, yet this lifesaving procedure resulted in a rise in Bethesda titer to ~800 against the vector-encoded pFVIII and nearly 700 to hFVIII. The formation of such high titer inhibitors with cross-reactivity to the human protein was surprising, given the well established ability to successfully use porcine FVIII products in human patients to bypass existing anti-hFVIII inhibitors [238-241]. Similarly, despite having no detectable inhibitors prior to transplant, the second animal receiving the higher FVIII-expressing cell dose developed titers of ~150 Bethesda units against the vector-encoded pFVIII following transplantation which also exhibited cross-reactivity to the human protein.

Following euthanasia of these animals, we performed a detailed tissue analysis to begin deciphering the mechanism whereby this novel MSC-based gene delivery produced its pronounced therapeutic effect at a systemic level. PCR analysis demonstrated readily detectable levels of MSC engraftment in nearly all tissues analyzed, including liver, lymph nodes, intestine, lung, kidney, omentum, and thymus. These molecular analyses thereby proved that it is possible to achieve widespread durable engraftment of MSC following transplantation

in a postnatal setting in a large animal model without the need for preconditioning/ablation, and in the absence of any selective advantage for the donor cells.

Confocal immunofluorescence analysis revealed large numbers of FVIII-expressing MSC within the synovium of the joints which exhibited hemarthrosis at the time of transplant, demonstrating (just as we had hoped/predicted) that the transplanted MSC possessed the intrinsic ability to home to and persist within sites of ongoing injury/inflammation, releasing FVIII locally within the joint, providing an explanation for the dramatic improvement we observed in the animal's joints. This finding is in agreement with prior studies [242], showing that local delivery of FIX-AAV to the joints of mice with injury-induced hemarthroses led to resolution of the hemarthroses in the absence of any detectable FIX in the circulation. While this finding provides an explanation for the reversal of the joint pathology present in these animals at transplant, it cannot explain the observed systemic benefits such as the cessation of spontaneous bleeding events.

Confocal analysis also revealed engrafted cells within the small intestine, demonstrating that MSC can still engraft within the intestine following postnatal transplantation, just as we had observed in prior studies in fetal recipients [232]. Given the ease with which proteins secreted from cells within the intestine can enter the circulation, future studies aimed at improving the levels of engraftment within the intestine have the potential to greatly improve the systemic release of FVIII. In addition to the intestine and hemarthrotic joints, significant levels of engraftment were also seen within the thymus of this animal. While the ability of the transplanted MSC to traffic to the thymus could clearly have important implications for the likelihood of long-term correction with this approach to hemophilia A treatment, additional studies are required to determine with which cells within the thymus these MSC are interacting to ascertain the immunologic ramifications of thymic engraftment.

The marked phenotypic improvement and improvement in quality of life we have observed in our studies, to date, in the sheep model thus support the further development of therapeutic strategies for hemophilia A and, perhaps, other coagulation disorders, employing MSC as cellular vehicles to deliver the required transgene.

8. MSC as anti-cancer gene delivery vehicles

As alluded to earlier, a large number of preclinical animal studies examining the differentiative potential of MSC isolated from a variety of adult tissues have also highlighted another interesting and clinically valuable characteristic of MSC; their ability to selectively navigate to sites of injury and/or inflammation within the body [192, 193, 243-247]. Once reaching these specific sites, the MSC then mediate repair both by engrafting and generating tissue-specific cells within the injured tissue (but contributing very little if at all to other tissues that are functionally normal [188-190]), and by releasing trophic factors that blunt the inflammatory response and often promote healing by activating the tissue's own endogenous repair mechanisms. While the mechanisms responsible for this trafficking to sites of injury

are currently not well understood, this observation has raised the exciting prospect of using MSC to treat a wide array of diseases in which inflammation plays a key role such as stroke [87, 88, 92, 248-255], rheumatoid arthritis [256], asthma [257-259] and allergic rhinitis [260], and both acute and chronic lung injury [261].

Cancer represents another condition in which there is a selective need for new cells created by the forming tumor, and a chronic state of insult/inflammation within the surrounding tumor microenvironment. Studies over the last several years have now revealed that MSC have the ability to “sense” this need for cells and the perceived injury to the tissue surrounding the tumor. As a result, both endogenous bone marrow- and adipose-resident MSC, as well as intravenously infused MSC, all appear to have the ability to efficiently migrate to the forming tumor, and contribute to the newly forming tumor “stroma” [191, 262-266]. Clearly, this may not seem ideal, since the MSC could, in fact, provide support to the growing tumor, potentially worsening the prognosis. Indeed, unraveling the role played by MSC within the tumor microenvironment is currently an area of active research [191, 192, 262-265, 267-269]. Irrespective of their role in the tumor’s health/biology, however, the ability of MSC to selectively traffic to and integrate into the tumor microenvironment can be viewed as a double-edged sword, since this ability has now been recognized to present a very powerful and unique means of selectively delivering anti-cancer gene products to tumor cells in vivo [270-274]. Four of the gene products which have thus far received the most attention are IL-2 [275, 276], IL-12 [277-284], IFN- β [270, 271, 285, 286], and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [287-298]. Unfortunately, the utility of these and many other biological agents that could be used for cancer therapy is often limited by both their short half-life in vivo and their pronounced toxicity due to effects on normal, non-malignant cells within the body. Using MSC to deliver these therapeutics promises to solve both of these problems, since the MSC can selectively migrate to the tumor site and release their therapeutic payload locally. This would be predicted to greatly increase the agent’s concentration within the tumor and significantly lower its systemic toxicity. In addition, by genetically modifying the MSC with viral vectors, the engrafted MSC will steadily release the therapeutic agent, allowing a single administration to result in long-lasting effects. Other studies have now provided evidence that MSC have the ability to not only selectively home to solid tumors [270, 271, 287, 299], but also to actively seek out metastases at sites far removed from the primary site of the tumor [271, 288, 290, 299, 300]. This ability has recently been proven to be of great therapeutic value in the treatment of lung metastases arising from both breast cancer and melanoma in a murine xenograft model [271, 299]. Given the difficulty and frequent lack of success using traditional approaches such as surgery, radiotherapy, and chemotherapeutic agents to treat tumors which are either highly invasive or prone to metastasis, this property of MSC will likely prove to be of great clinical value in the near future.

One form of cancer for which the use of MSC is receiving a great deal of attention is glioblastoma multiforme (GBM). GBM represents the most common form of malignant glioma. Despite decades of research and many advances in the treatment of this disease with con-

ventional surgery, radiotherapy, and chemotherapy, there is no cure, and the current prognosis is abysmal, with a median survival of only 6-18 months. The failure of current therapies to cure this disease arises predominantly from the highly invasive nature of this cancer and the inability of these agents to effectively target tumor cells which have disseminated into the normal parenchyma of the brain, at sites distant from the main tumor mass. Given the ability of MSC to home to tumors and their ability to track to metastases throughout the body, gene-modified MSC are receiving a great deal of attention as a possible therapy for GBM. Studies have now shown that MSC migrate through the normal brain parenchyma towards gliomas and appear to possess the uncanny ability to track microscopic tumor deposits and individual tumor cells which have infiltrated the normal brain parenchyma [276, 282, 289, 290, 301-310]. While these migratory properties are certainly interesting, even more exciting are the dramatic therapeutic benefits these same studies have shown, with reduction in tumor size, and pronounced improvements in survival. It is important to note that, in most of these studies, MSC were used as the sole therapy, and definite benefits were observed. In the clinical setting, the current plan is to use gene-modified MSC as an adjunct after surgical resection. In this scenario, the vast majority of the tumor mass would be surgically removed, and the MSC would then be transplanted, in the hopes that they would then remove the residual malignant cells at the site of the tumor, and then hunt down and eliminate any invasive tumor cells that have migrated away from the site of the primary tumor. In this context, one would imagine that the therapeutic benefit of the MSC will likely be even more pronounced, since their anti-tumor effects could be focused only on the small number of residual tumor cells that evaded removal during surgery. The remarkable success seen in studies aimed at treating GBM, one of the most devastating forms of cancer, thus serve to highlight the tremendous potential MSC harbor as gene delivery vehicles for the treatment of many forms of cancer for which current therapeutic strategies are ineffective.

9. Conclusions

Numerous investigators around the globe have now provided compelling evidence that MSC from a variety of tissues possess a far broader differentiative capacity than anyone would have foreseen at the time Friedenstein originally described his bone marrow-derived stromal cells. Extrapolating the work thus far on using MSC to deliver FVIII to treat hemophilia A, and the rapidly growing number of studies showing the tremendous potential of MSC as anti-cancer gene delivery vehicles, and combining this with the relative ease with which MSC can be isolated, propagated in culture, and modified with a variety of viral-based vectors, and their intrinsic ability to seek out sites of injury/inflammation within the body, one can readily see why MSC are widely viewed as being ideally suited not only as cellular therapeutics, but as vehicles to deliver gene therapy vectors to numerous tissues in the body, thus promising to provide a permanent cure for a diverse range of diseases.

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Cancer Gene Therapy – Key Biological Concepts in the Design of Multifunctional Non-Viral Delivery Systems

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Additional information is available at the end of the chapter

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1. Introduction

The importance of gene therapy strategies for the treatment of malignancies is highlighted by the fact that there are (at the time of writing) 823 cancer gene therapy clinical trials worldwide that are actively, or have yet to begin recruiting patients (www.clinicaltrials.gov - accessed November 2012). The potential of the delivery of genetic material for therapeutic purposes has long been recognised, but to this point, has yet to be successfully translated. Strategies that have proved promising in the *in vitro* setting have stumbled when exposed to the complexities of the *in vivo* environment. Classically involving the delivery of plasmid DNA (pDNA) that encodes a therapeutic protein product, the field of gene therapy has evolved to encompass not only delivery of therapeutic DNA, but also micro- (miRNA), short hairpin- (shRNA) and small interfering RNAs (siRNA) and oligodeoxynucleotides (ODNs) [1]. Despite the evolution of the technology for altering the genotype of target cells and tissues, the problem of overcoming the biological barriers that limit the efficacies of these technologies remains. These barriers exist at both systemic and local levels. To date, the only approved nucleic acid-based treatments for clinical use are an antisense ODN for the treatment of cytomegalovirus retinitis [2], and pegaptanib sodium (Macugen), an RNA aptamer targeted against VEGF-165 and used to treat age-related macular degeneration [3]. This chapter will focus on the biological barriers faced by non-viral vectors for gene therapy, strategies that have been employed to overcome these barriers, and will conclude by documenting the state of the art technologies being used to propel non-viral gene therapies forward.

1.1. Non-viral gene therapy

Delivery of genetic material for therapeutic use from virus-like particles has received considerable attention, and has generated extensive knowledge. The molecular evolution of viruses

over the aeons has produced DNA-delivering organisms of incomparable efficiency. The use of 'gutted' viruses that lack virulence properties and replicative capacity is the most efficient method of genetic material delivery [4], and modified viruses have been used extensively in gene therapy; commonly employed viruses include adenovirus, retrovirus, vaccinia virus and herpes simplex virus [5]. The allure of viral gene therapy was hindered however, when a clinical trial patient died four days after receiving adenoviral therapy for treatment of ornithine transcarbamylase deficiency [6]. The negative press that this generated, along with other disadvantages of viral gene therapy (including generation of immune response, possibility of proto-oncogene activation, production costs, and limitations in deliverable gene size) have necessitated the generation of alternative gene therapy strategies [7].

Despite some success when naked DNA has been delivered *in vivo* (naked pDNA has been effectively delivered to the liver in mice and rats [8] by tail vein injection), pDNA for gene therapy is conventionally delivered complexed with materials with suitable physical characteristics. pDNA's hydrophilicity and anionic nature impair the uncomplexed molecule's passage through the lipophilic plasma membrane [1,9,10]. Non-viral gene therapy strategies usually involve wrapping of the nucleic acid to be delivered in a protective envelope that neutralises the negative charge of the DNA. A range of compounds has been used to envelop pDNA, including cationic lipids, polymers and peptides.

Cationic lipids were among the first compounds complexed with pDNA for non-viral gene delivery. Felgner reported that N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) formed lipid-DNA complexes based on the interaction between the positively charged lipid and the negatively charged phosphates of the DNA. The lipoplexes so-formed were capable of delivering DNA to cells *in vitro* [11]. Numerous cationic lipids have since been reported to neutralise, condense and encapsulate pDNA, including dioctadecylamidoglycylspermine (DOGS) [12], [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane] (DOTAP) [13] and 3β [N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) [14]. Variations on a theme, these lipids behave similarly to innate biological lipids [15]. The addition of co-lipids such as cholesterol and dioleoylphosphatidylethanolamine (DOPE) can improve transfection efficiency [16]. Recent developments in the field have seen a 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC):cholesterol liposome with folate electrostatically-associated used to deliver HSV-tk suicide gene therapy to SCC-VII xenografts, which resulted in considerable tumour growth delay [17]. In a magnetofection method, intravenous delivery of superparamagnetic iron oxide lipid nanoparticles in combination with an Nd-Fe-B magnet placed externally in the tumour locality resulted in improved IGF-1R shRNA delivery to A549 xenografts [18].

Cationic polymers have been used to condense and deliver genetic material, including poly(l-lysine) (PLL), polyethylenimine (PEI), chitosan and polyamidoamine (PAMAM) [7]. PLL incorporated into a spider silk-based nanoparticle with a tumour-homing peptide was recently reported to deliver a luciferase plasmid to MDA-MB-231 xenografts following intravenous administration [19]. A novel triblockpoly(amido amine)-poly(ethylene glycol)-poly(l-lysine) (PAMAM-PEG-PLL) nanocarrier successfully delivered Bcl-2 siRNA and elicited knockdown of the same in A2780 ovarian carcinoma cells *in vitro* [20]. Heparin-PEI nanogels

were used to reintroduce heparin sulphate 6-O-endosulfatase 1 (HSulf-1) to ovarian SKOV-3 xenografts, which resulted in anti-angiogenesis, induction of apoptosis and suppression of cell proliferation [21], while a PEI-poly(hydroxyethyl glutamine) (PEI-PHEG) copolymer successfully delivered pGL3 pDNA by intratumoural administration to Lewis Lung Carcinoma xenografts in C57BL/6 mice [22].

Cationic peptides that are capable of neutralising, condensing and wrapping pDNA have also been used as non-viral delivery vehicles [23]. These cell-permeating peptides were designed to interact with cell membranes similarly to viral fusion proteins. GALA, a synthetic peptide designed to interact with lipid bilayers at an acidic pH, was observed to aid in the delivery of DNA to cells [23]. A derivative, termed KALA, through presence of positively charged lysine residues, is capable of condensing and delivering DNA unaided [24], and improved gene delivery ten-fold in hepatoma [25] and also in HEK293T and HepG2 cells [26]. The cell-penetrating peptide TAT and fusogenic peptide HA2 were used to improve pDNA delivery by gelatin-silica nanoparticles [27]. Recent developments in the field have seen the development of multi-domain peptidic biomimetic vectors tailored to overcome the various biological barriers that gene delivery vehicles encounter *in vivo*, including degradation by serum nucleases, endosomal entrapment and nuclear localization. One such designer biomimetic vector was used to deliver tumour-related apoptosis inducing ligand (TRAIL) [28] and inducible nitric oxide synthase (iNOS) pDNA to ZR-75-1 breast cancer cells *in vitro* [29].

Non-viral strategies for gene therapy have several advantages over traditional viral approaches, including reduced cost and ease of large-scale production, as well as avoidance of the virulence commonly associated with viral delivery. However, non-viral gene delivery systems suffer from lower potency of transfection ability, resultant of lower ability to traverse the various obstacles faced upon administration [7].

2. Extracellular barriers to gene delivery

2.1. The skin

The most fundamental barrier that the human body possesses is its skin. The stratum corneum is the skin's outermost layer, and provides an imposing barrier to gene delivery [30]; the densely packed cornified cells of this layer protect the body from a range of foreign material. The skin is not a commonly used route for gene therapy approaches, but it is an attractive route for local targeting of dermatological ailments [31]. However, skin nucleases, and in particular DNase 2, active at the skin and in the stratum corneum, degrade topically-applied nucleic acids [32]. An appropriate and potent delivery mechanism could open the door to gene therapy strategies for the treatment of skin conditions and malignancies, including xeroderma pigmentosum (a cancer-linked disorder that has shown promise in pre-clinical gene therapy approaches [33]), when replacement of the defective XPC gene in keratinocytes would be therapeutically beneficial [34].

Introduction of micron-sized pores to the skin using minimally-invasive silicone micro-needles allowed for the delivery of a 'non-viral gene vector-mimicking' charged fluores-

cent nanoparticle [35,36]. A needle-free injection device successfully delivered luciferase pDNA to porcine skin, resulting in higher protein expression than conventional hypodermic needle administration [37], while subcutaneous melanoma xenografts were targeted for gene therapy using a hybrid electro-microneedle; delivery of 20 µg interleukin-12 pDNA to the skin, followed by eight 50 ms pulses delivering 70V/0.5 cm from the electrode to improve transfection resulted in a significant improvement in survival of tumour-bearing mice [38]. Most recently, researchers from Northwestern University reported the generation of siRNA-carrying nanoparticles (spherical nucleic acid nanoparticle conjugates (SNA-NCs)) that are capable of penetrating nude mouse and human skin, whilst maintaining their RNA interference potential [36]. The nanoparticles comprise gold cores surrounded by a dense shell of highly oriented, covalently immobilised siRNA and could be delivered topically, avoiding the need for disruption of the skin. As the skin is unmolesed, the authors propose that the miniscule nature of the SNA-NCs permit dermal crossing, a theory that is currently under investigation.

2.2. Barriers to systemic gene therapies

Needle-administered systemic therapeutics bypass the skin, but encounter further extracellular barriers before reaching their site of action. The various administration routes (intravenous, -muscular, -ocular, -nasal) present their own unique impediments to nucleic acid delivery. Intravenously- [39] and intramuscularly-administered [40] therapies are subject to nuclease degradation from the point of entry. Conversely, naked uncomplexed anti-respiratory syncytial virus (RSV) siRNA was almost as effective as that complexed with TransIT-TKO transfection reagent when nasally-administered in mice [41], suggesting that nasally-administered gene therapies may not be as prone to nuclease insult. The compartmental nature of the eye, and ease of access to it simplifies avoidance of similar barriers in ocular gene therapy delivery [42]. pDNA complexed with poly(D,L-lactic-co-glycolic) acid (PLGA) and dimethyldioctadecylammonium bromide (DDAB) produced nanoparticles capable of traversing one of the most inhospitable of barriers, the gastric mucus [43]. For simplicity, this chapter will focus on the barriers faced by intravenously delivered therapies, as this route has the potential to target almost all tissues of the body.

The complexing of DNA into lipo- or polyplex nanoparticles in non-viral delivery can effectively protect the pDNA from nuclease degradation [44] (although, paradoxically, cationic and anionic lipoplexes can hinder pDNA delivery by electroporation [45]). Whilst in the circulation, however, non-viral agents can be subject to non-specific binding by serum proteins, which can result in aggregation or dissociation of nanoparticles, resultant of the generally positive charges of the nanoparticles and the negative charge of circulatory proteins [1]. Positive charge is essential to ensure interaction of the nanoparticle with its target cell, however the mononuclear phagocytic system (MPS) eliminates foreign hydrophobic particles from the circulation [7] by opsonisation. The MPS was neutralised in mice by pre-treatment with polyinosinic acid (a synthetic nucleic acid strand) before therapeutic measles virus treatment; this led to competitive inhibition of the scavenging of particles by macrophages, and improved virus delivery to and efficacy at SKOV3 xenografts [46]. Aggregation

of nanoparticles can cause embolization of microvessels, and non delivery of the therapeutic to target [1].

The differential in ionicity between gene therapy formulation and the extracellular space poses another obstacle for nanoparticles, which can lead to colloidal instability [44]. The issue of non-specific interaction between nanoparticles and plasma proteins has been addressed by the coupling of hydrophilic molecules to the nanoparticle. The most commonly employed candidate is poly(ethylene glycol) (PEG), whose anionicity has led to reduced aggregation and improved transfection ability [47]. PEG has been incorporated into a myriad of non-viral gene therapy strategies. Recently, polyacridine peptide nanoparticles were PEGylated and found to persist in the mouse circulation for up to nine hours, compared to non-PEGylated nanoparticles, which were inactive within five minutes [48], while biodegradable dextran nanogels were PEGylated and analysed for their siRNA delivering prowess [49]. Particulate gene therapies are also subject to entrapment by the mononuclear phagocyte system (reticuloendothelial system - RES), when they are captured and held in the spleen or liver [50], which was responsible for the inactivation of adenoviral vectors that have been used as viral gene therapeutics [51]. Avoidance of non-specific biomolecular interaction, referred to as 'stealth' [1], is a prerequisite for successful gene delivery. Functionalisation of non-viral gene therapies with agents such as PEG to facilitate RES avoidance will be discussed in subsequent sections.

Assuming a gene therapeutic persists in active form in the circulation and the target tissue is reached, extravasation from the circulation is imperative. The architecture of normal vasculature ensures that transport of macromolecules out of the circulation is difficult. One characteristic of tumour vasculature, however, is its propensity to leakiness, an attribute that can be exploited by gene therapies. It is unsurprising that siRNA lipoplexes that target RAN GTPase were delivered more effectively, and evoked more impressive anti-tumour effects in highly vascularised xenografts than in xenografts with poorer vascularity [52]. The leaky vessel phenomenon, known as the enhanced permeability and retention (EPR) effect, has been utilised to enable the delivery of pDNA-containing particles in various malignancies [53]. The utilization of EPR will be further discussed below. The angiogenic tumour vasculature was itself targeted in a murine dorsal air sac assay; siRNA targeting Ago2 was complexed into cationic liposomes and intravenously administered. The authors successfully delivered the interfering RNA to the angiogenic vessels, and reported tumour regression, presumed to be resultant of anti-angiogenesis in their model [54].

Perhaps the most intimidating vascular obstacle that a gene therapy can face is the blood-brain barrier, where tight junctions between endothelial cells of the capillaries limit the passage of molecules much more than at other capillary sites in the body. One of the most exciting techniques available to the gene therapy researcher is the use of ultrasound-targeted microbubble destruction (UTMD); nucleic acid contained within a gas-filled microbubble is administered, before exposure to ultrasonic waves at a frequency that exceeds the resonance frequency of the microbubbles, causing their destruction and leading to increased capillary and cell membrane permeability [55]. This technology was used to deliver pDNA for

the green fluorescent protein (GFP) reporter gene across the mouse blood-brain barrier [56], and presents new possibilities for overcoming this most daunting of circulatory barriers.

2.3. Cellular barriers to gene delivery

2.3.1. *The cell membrane and endocytosis*

Specific targeting of gene vectors to ensure delivery to the target tissue will be discussed in a subsequent section. The nanoparticle's nucleic acid cargo determines the site to which delivery is required; plasmid DNA must be delivered to the nucleus to affect transcription, while siRNA need only reach the cytoplasm to interfere with translation [57]. The most elemental impediment to entry into the animal cell is the lipid bilayer membrane. The cell membrane can be breached using physical means in certain circumstances to allow delivery of naked pDNA. These methods include electroporation (local destabilization of the cell membrane using an electric pulse), sonoporation (membrane destabilization using ultrasound) or laser irradiation (introduction of transient pores in the membrane using a lens-focussed laser beam). The application of these methods is limited, however, by inaccessibility to most tissues [58].

Condensation and neutralization of nucleic acids into nanoparticles abrogates the two fundamental properties of pDNA that preclude its cellular entry, namely its large size and negative charge [1]. Particles of excessive size can aggregate and cause embolization of narrow capillaries, as mentioned above. Nanoparticles for gene delivery tend to be sub-200 nm for this reason. However, *in vivo* delivery of fluorescently labelled liposomes of up to 400 nm diameter has been reported [59]. Particle size also appears to dictate the pathway that performs internalization of complexes; 200 nm particles have entered cells by clathrin-dependent routes, 300 nm particles by caveolae-mediated pathways [7]. Optimization of the net charge (or zeta/ ζ potential) of delivery vehicle/nucleic acid complex of lipid/polymer/peptide and pDNA complexes is achieved by the electrostatic interaction between the negatively charged phosphate residues present in the pDNA and the positively charged nitrogen in the vehicle. The net charge of the resultant particle can be increased by increasing the vehicle (nitrogen) to pDNA (phosphate) ratio (known as N:P ratio) [1]. The negative charge of serum proteins can thwart the therapeutic potential of nanoparticles; this can be overcome by increasing the N:P ratio of complexes above that sufficient to condense the pDNA. Altering the net charge of the nanoparticles can significantly alter the array of plasma proteins that interact with the particles. Similar liposomal particles with charges of -9.0, -11.4 and -27.4 were incubated with human plasma, and the interacting proteins were identified; 117 proteins were found bound to particles of all three charges, while 12, 6 and 15 plasma proteins interacted uniquely with the three particle charge types respectively [60]. Kim and co-workers reported that hyperbranched polysiloxysilane nanoparticles with a moderate positive charge (46 mV) were more efficient gene delivery agents than analogous particles with a high positive charge (64 mV) [61]. Clearly, nanoparticle size and charge are parameters that require optimization for appropriate cell membrane breaching. Phosphonium-based vectors (as opposed to nitrogen-based) are also being explored for their gene delivery

potential; preliminary studies have revealed that phosphonium-based vectors condensed DNA at lower charge ratios than corresponding nitrogen-based vectors [62].

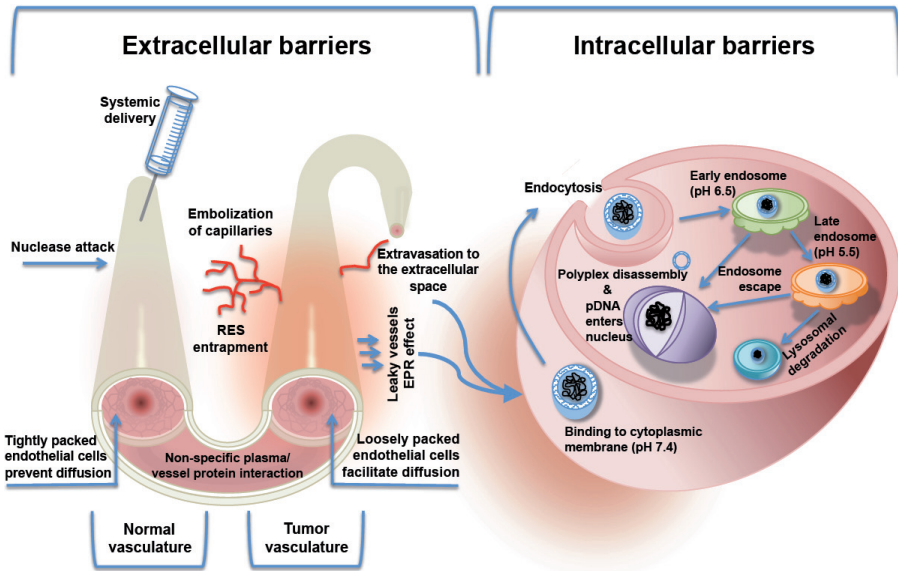


Figure 1. Summary of the extra- and intracellular barriers faced by non-viral gene therapies following systematic delivery. Based on [1].

Electrostatic interaction between the cationic nanoparticle and the anionic cell membrane that facilitate association between the cell and nanoparticle was assumed to result in endocytosis of the nanoparticle, although the machinery of internalization appears to be material- and cell type-dependent. Endocytotic access to cells is by pinocytosis in the majority of cases, rather than by phagocytosis [9]. A mechanism of endocytosis was clarified by Payne and colleagues, who followed the intracellular trafficking of PEI- and LipofectamineTM-complexed nucleic acids in mammalian cells, and reported that endocytosis relied upon cell surface heparin sulphate proteoglycans (HSPG) and was dependent on dynamin- and flotillin, rather than clathrin- and caveolin-dependent mechanisms [63]. On the other hand, nanoparticles formed from pDNA complexed with PEG-CK₃₀ were endocytosed after interaction with cell surface nucleolin, a process that was reliant on the activity of lipid rafts [64]. A recent study reported by a team at the University of Groningen very elegantly showed lassoing of PEI- and LipofectamineTM-complexed pDNA by syndecan- and actin-rich filaments of HeLa cells, presumed to be filopodia and retraction fibers; the nanoparticles then ‘surf’ along the filopodia, or the filopodia are retracted toward the cell body, thereby facilitating HSPG-mediated endocytosis [65].

2.3.2. Endosomal escape

The result of endocytic cellular entry is endosomal entrapment. Endosomes are a range of membrane-bound organelles that include early, late and recycling endosomes that are responsible for the short-term storage and sorting of endocytosed materials, including macromolecules and pathogens (including viruses). Once material is endocytosed, it is either evicted from the cell by the recycling endosome, or the complex process of endosome maturation ensues, late endosomes fuse with lysosomes, and active degradation of endosome cargoes occurs [66]. Macromolecules that are unable to escape the endosome are bound for lysosomal degradation.

The mechanism of endosomal escape by non-viral vectors is dependent on the complexing material used. Cationic lipids appear to interact with the anionic endosomal membrane, resulting in ion pair formation and consequent transformation to inverted hexagonal phase (H_{II}), causing disruption of the endosomal membrane. Alternatively, an inversion of the endosomal membrane as a result of electrostatic interactions has been proposed, which would instigate nucleic acid cargo being deposited in the cytoplasm. When polymeric materials are used to complex pDNA, the polymers themselves absorb protons in the endosome (proton-sponge effect), leading to chloride ion influx, increased osmotic pressure and water flow into the endosome, resulting in endosome rupture [7].

The fusogenic lipid DOPE is frequently used as a co-complexing agent due to its inherent ability to facilitate endosomal escape; conformational change from lipid bilayer to an inverted hexagonal structure is triggered by the sub-physiological pH of the endosome, and causes endosomal membrane disruption and escape of nucleic acid cargo [67]. Viral membrane proteins have provided inspiration for the non-viral gene delivery researcher. Influenza viruses escape the endosome with the help of hemagglutinin A2 (HA2), while the adenoviral protein, penton, assists adenovirus endosome escape. Glycoprotein H from herpes simplex virus induced 30-fold improvement of transfection by lipoplexes in human cell lines [68]. Conformational changes of these proteins consequential of the acidic environment in the endosome facilitate viral particle escape from the endosome; the more hydrophobic conformation that they adopt at low pH permits membrane fusion and disruption [1].

Synthetic fusogenic peptides are increasingly being used to improve transfection in non-viral systems. The conformational status of GALA is responsive to pH, adopting alpha-helical status in acidic environments, and in that sense, mimics the endosomal escape route favoured by viruses [69]. Derivatives of GALA such as GALAdeIE3, YALA [70], and KALA [24] have all shown promise as endosome escaping agents. Similarly, two endosomal escape peptides, INF7 and H5WYG, improved endosome escape of PEG-based vectors by up to 100-fold [71].

2.3.3. Nuclear envelope penetration

The final obstacle faced by pDNA gene therapies is the nuclear envelope, a barrier punctuated with nuclear pores impermeable to molecules greater than 70 kDa, or roughly 10 nm in diameter [72]. Liposomal fusion with the nuclear membrane that facilitates direct cargo

transfer from vector to nucleus has been reported [73]. Mitotic division temporarily disrupts the nuclear membrane's barrier properties, which can allow pDNA transgene entry [9]. The nuclear pores can be more actively targeted for penetration by the use of nuclear localization signalling (NLS) peptides or DNA targeting sequences (DTS). NLSs are short clusters of basic amino acids (such as lysine) that bind to importins, receptors that facilitate cytoplasm-nuclear transport [74]. Active transport of macromolecules through nuclear pore complexes causes expansion of the pores to approximately 30 nm in diameter [75]. The nuclear localization peptide SV40 from Simian virus 40 was used to improve the delivery of luciferase gene-carrying liposomes to neuroblastoma cells [76], while NLSs from adenovirus E1a, the transcription factor c-myc, mouse FGF3, and the DNA repair protein PARP have all been used to guide transgene delivery to the nucleus [74]. Some of the recently employed nuclear envelope penetration strategies are summarised in Table 1.

3. Evading the immune system

As mentioned previously, although viruses are masters of nucleic acid delivery, alternative delivery mechanisms are being sought to avoid the pitfalls associated with viral systems. Fundamentally, viruses remain foreign pathogens, agents that the human body has evolved to protect itself from. Of the commonly employed viral vectors, adenoviral, adenovirus-associated vectors and lentivirus vectors all produce immune responses in mice and humans, with antibodies often being produced against both the packaging vector as well as the transgene product. Exposure to viral particles triggers the adaptive immune response. Pinocytosis of viral particles by immature dendritic cells elicits maturation of the dendritic cells into mature antigen-presenting cells that present antigens in major histocompatibility complexes (MHCs). Activation of T cells by antigen presentation leads to both the destruction of the antigen-presenting cells, and the recruitment and activation of B cells, responsible for antibody production [84].

Attempts to avoid provocation of immunologic responses have been made by the viral gene therapist that include deletion or nullification of viral coding genes and elimination of pathogenic genes, or use of targeting strategies to ensure avoidance of the immune cells. Additionally, pharmacological immunosuppression has been used extensively to avoid the neutralization of various viral gene therapy strategies [85].

3.1. DNA-mediated immune responses

It is generally accepted that non-viral gene therapy strategies elicit fewer immune responses than their viral counterparts, although certain facets of non-viral complexes mark them as targets for immune system intervention [86]. An early report into immune responses induced by non-viral gene therapy revealed cytokine induction (TNF α and IL-1 β) by PEI/DNA complexes; the extent of immune induction was determined by the route of delivery, aerosol proving less detrimental than intravenous [87]. In mice, lipoplex administration evoked complement activation and induction of IFN- γ , TNF- α , IL-6, and IL-12. These effects

NLS	Sequence	Summary	Result	Ref
TAT	Ac-GCGYGRKKRRQRRG-NH ₂	PEG-based vector with DNA binding peptide	Up to 15-fold increase in CHO cell transfection	[77]
NLS-1	DPKKKRKV	Inclusion of NLS peptides in Lipofectamine liposomes for transfection into human and rat mesenchymal stem cells	Roughly two-, four- and six-fold enhancement of luciferase expression respectively	[78]
NLS-2	DPKKKRKVDPKKKRKV			
NLS-3	DPKKKRKVDPKKKRKV-DPKKKRKV			
I-NLS	Iodinated-PKKKRKV	Iodinated NLS was complexed with pDNA and PEI. Luciferase transfection was assessed in MCF-7 breast cancer cells	130-fold improvement in transfection compared to absence of NLS. Iodination improved nuclear localization	[79]
Human surfactant protein C promoter	318 nucleotides PCR amplified from genomic DNA	Sequence cloned into promoterless plasmid and microinjected into cytoplasm of MLE-12 cells.	Fluorescent <i>in situ</i> hybridization revealed nuclear localization in 25-30% of injected cells compared to control (0%). Specific to alveolar type II epithelial cells	[80]
Triamcinolone acetonide (TA)	N/A	TA was conjugated to PEI (various molecular weights) and nuclear localization determined	Low molecular weight PEI/TA efficiently targeted the nucleus	[81]
Dexamethasone	N/A	Polyplexed (PEI) with pLuciferase	10 – 100-fold increase in transfection efficiency	[82]
Trans-cyclohexane-1,2-diol	N/A	Amphipathic alcohol that collapses nuclear pore cores allowing macromolecule uptake	Improved Lipofectamine 2000-mediated gene transfection to 293T cells <i>in vitro</i> , but was not reproducible <i>in vivo</i>	[83]

Table 1. Recent strategies employed to aid the delivery of non-viral gene therapies to the nucleus.

were independent of N:P ratio or the cationic lipid complexed with the pDNA [88]. Although observed immune responses tend to be dose-dependent, dose reduction to avoid immune induction consequently also lessens the transfection ability of the complexes, stressing the narrow therapeutic index of non-viral gene therapies [89].

It is well established that immune responses in non-viral therapies is resultant of the presence of unmethylated CpG motifs in the bacterial backbone of the plasmid. In mammals, roughly 75% of CpG motifs are methylated to 5'-methylcytosine, whereas in bacteria they are usually unmethylated [89]. Recognition of unmethylated CpG motifs by Toll-like receptor 9 on immune cells causes activation of mitogen-activated protein kinases and NF-κB [90]. As

well as eliminating an immune response, evidence exists to suggest that removal of unmethylated CpG motifs can increase the duration of transgene expression [91,92]. PEI-based delivery of CpG-rich pDNA was associated with a reduction in lung compliance, while delivery of CpG-diminished pDNA was not [93]. Furthermore, methylation of CpG motifs in pDNA largely reversed the immunostimulatory activity of lipoplexes and polyplexes in C57BL/6 mice [94].

Numerous strategies have been investigated to abrogate immune responses upon non-viral gene therapy administration. Liu and colleagues encapsulated various anti-inflammatory agents into DOTAP/pLuciferase liposomes, and termed the resultant complex a 'safeplex'. Safeplexes carrying dexamethasone, prednisone, indomethacin, tetrandrine and gliotoxin inhibited TNF α expression compared to that seen in the absence of anti-inflammatory. Importantly, the complexing of dexamethasone into the safeplex did not affect the complex's ability to deliver its pDNA cargo [89]. Delivery of oligonucleotides to inhibit cytokine (NF κ B) translation using non-viral carriers has also been proposed as a mechanism of counteracting the host's immune response to non-viral therapies [95].

Lipid-protamine-DNA complexes (LPDs) were used to deliver a PCR amplicon of the luciferase gene rather than a bacterial plasmid containing the luciferase gene. Luciferase translation was as efficient from the PCR amplicon as from the plasmid when both were complexed with LPD, but the immune response evoked by the PCR fragment complex was three-fold less potent than that evoked by the plasmid complex (determined by TNF α and IL-12 expression) [96].

The immune response can be avoided by the removal of unnecessary bacterial DNA that contains the immunological CpG motifs, bacterial origin of replication, as well as genes for plasmid antibiotic resistance that are not required for transgene expression. First reported in 1997, minicircles are gene delivery vehicles that lack prokaryotic nucleic acid, and were produced by the thermo-responsive activity of λ integrase [97]. Minicircles were more potent reporter gene deliverers than their parental pDNA in melanoma and colon carcinoma cell lines by lipofection and electroporation [98]. Minicircles complexed with PEI also delivered the GFP gene more potently than similarly complexed pDNA [99]. The potency of minicircles has been improved by tethering minicircle liposomes to the TetR nuclear targeting device [100].

A further improvement on the immunologically inert minicircles has recently been mooted. Tightly-wound miniknot vectors are the result of DNA minicircle treatment with DNA topoisomerase II, and are proposed to be more resistant to physical damage (strand breaks) that can linearise (thereby reducing/removing efficacy) pDNA and minicircles. DNA delivery methods such as aerosol inhalation, jet-injection, electroporation, particle bombardment and ultrasound DNA transfer can subject DNA to stresses that might cause damage [101].

3.2. Carrier-mediated immune responses

It is important to note that immune responses to non-viral gene therapy are not solely resultant of bacterial CpG motifs. An impressive study from Kyoto University highlighted the

immune responses that can be generated by liposomes. Using CpG-free pDNA in lipoplexes, the authors demonstrated activation of IFN β , TNF α and IL-6 in macrophages from TLR9 knockout mice. The extent of the immune response (as determined by *in vitro* cytokine induction) was dependent on the cationic lipid content of the complex. The reactions elicited by the cationic lipids can be summarised as Lipofectamine 2000 > Lipofectamine Plus > DOTMA/DOPE > DOTMA/cholesterol [102]. The inertness of DOTMA/cholesterol as delivery vehicle was supported further *in vivo*, when CpG-free pDNA lipoplexes provoked no IL-6 or IFN β induction after intravenous injection in mice [103]. The targeting of nucleic acid cargoes to specific cells/tissues (to be discussed shortly) could also remedy the immune response by preventing the transfection of non-target cells, and in particular, the antigen-presenting cells [85].

The continuing evolution of the non-viral gene therapy field has led to the development of transposon-based delivery strategies, including Sleeping Beauty, Tol2, and piggyBac. These systems appear to deliver DNA as efficiently as viruses, and provoke extended transgene product expression, whilst maintaining the low immunogenicity and other risk factors associated with viral gene delivery [104]. It is anticipated that the momentum of non-viral gene therapy research will lead to the development of vehicles and cargoes that will rival the viral gene therapy field.

4. Targeting in non-viral systems

The optimal gene delivery vector will protect its payload from degradation in the circulation, enable extravasation from the bloodstream, traverse cellular membranes, facilitate endosomal disruption to deliver the payload to either the cytoplasm, or if necessary, transport to the nucleus. The optimal vector should also be non-immunogenic, as discussed above. Design of such vectors obviously presents a huge challenge. Furthermore, for vectors that accomplish extra- and intracellular barrier and immune system avoidance, there is the added layer of complication that targeting presents. Frequently in cancer studies, the payload to be delivered is a therapeutic designed to over-express a protein or knockdown a gene to manifest an anti-cancer effect. In order to spare normal tissue damage, widespread toxicity, and to achieve a clinically viable therapeutic product, targeting has become an essential in the quest for a perfect vector.

4.1. Enhanced permeation and retention effect

Exploitation of the tumour microenvironment presents an obvious option in the targeting strategies employed by many delivery systems. The enhanced permeation and retention effect (EPR), which was mentioned briefly above, is a phenomenon whereby there is defective architecture in blood vessels, extensive angiogenesis, increased vascular permeability and an impaired function of the mononuclear phagocytic system [105-107]. The consequences of these tumour-specific physiological changes is that macromolecules > 40 kDa selectively 'leak' out of the blood vessels and extravasate into the interstitial tumour tissue [108,109].

Particle size is an important factor for utilizing the EPR effect. Studies have shown that nanoparticles up to 400 nm in diameter can permeate across tumour vessels [59,110]. However, circulation times can also play a key role in successful tumour transduction, with a minimum of 6 h required for the EPR effect to occur [111]. The EPR effect has been exploited not only in chemotherapy drug design but also in gene delivery. In one such example, polyglycerolamine (PG-Amine) dendrimers were complexed with siRNA and delivered intravenously to mice bearing luciferase tagged mammary tumours; after 24 h, there was a 69% reduction in luciferase activity [112]. The authors also reported that there was clear evidence of accumulation of the complexes in the tumours but not in any other organs, which can be attributed to the EPR effect. In order for continuous knockdown of luciferase, it was determined that injections would be needed every four days [112].

There are however, a number of problems if the EPR effect is the sole mechanism for targeting in cancer gene therapy. One such problem is tumour size. Tumours that are larger than 1 cm in diameter develop hypoxic regions that are characterised by a lack of blood vessels, therefore the EPR effect becomes redundant in these resistant regions. Evans blue dye and albumin were used to generate a synthetic macromolecule delivery of which showed that although selective, in tumours larger than 3 cm, the dye accumulated solely in the peripheral regions and not in the central core [113]. One method to increase the EPR in solid tumours is through the use of nitric oxide donors in combination with macromolecular delivery. Endogenous nitric oxide (NO) has an effect on blood flow, angiogenesis and metastatic potential [114-116]. From a therapeutic perspective there are conflicting reports as to whether it is better to enhance or inhibit nitric oxide within tumours [117]. From an enhancement perspective, studies within our own group using iNOS (inducible NO synthase) gene therapy have shown the cytotoxic and radio-chemo sensitizing effects through the generation of μM levels of NO [118,119]. The controlled generation of high levels of nitric oxide as the gene therapy has several anti-cancer advantages, including a genotoxic effect through oxidation, deamination and alkylation of DNA, reduction of the efficiency of DNA repair proteins such as Poly ADP Ribose Polymerase, inhibition of the transcription of hypoxia inducible factor and the anti-apoptotic factor NF- κB which reduces many other pro tumourigenic factors such as MMP1, 3, 9, VEGF, survivin and BCL2 [120]. Derivatives of NO such as peroxynitrite (ONOO) have also been shown to potentiate the EPR effect. Wu and colleagues showed that this was also linked to activation of MMPs, which are known to enhance vascular permeability and angiogenesis through the degradation of matrix proteins [121]. One of the main ways of enhancing the EPR effect is through NO donors, which have been utilised in combination with chemotherapy drugs. For example nitroglycerin has been delivered with vinorelbine and cisplatin in patients with non-small cell lung cancer in a randomised phase II trial. Results showed a response rate of 73% in those patients that received the nitroglycerin plus chemotherapy, compared to 42% in the chemotherapy only arm. The improved effects were attributed to the known anti-cancer effects of NO and an improvement in drug delivery to the tumour tissue, i.e. the EPR effect [122]. With respect to gene therapy, the delivery of the iNOS gene would most certainly enhance the EPR effect in solid tumours although consideration must be given to the amount of NO generated. As gene expression is dependent on successful nuclear transport of the plasmid, it would be very difficult to pre-

dict and indeed control. With NO gene therapy, it is therefore essential to target the expression of the gene to the target tissue to gain the maximum therapeutic benefit.

Another problem in EPR targeting comes about with what is commonly termed the 'PEG dilemma'. This is essentially a trade-off between circulation time and efficacy of nucleic acid delivery. Many nanoparticles are PEGylated to increase circulation time, avoid clearance by the reticuloendothelial system (RES) and evade an immune response. As previously stated, if the EPR effect is to be exploited in solid tumours, a long circulation time is needed and PEG represents a possible solution. However, the physiochemical properties of many delivery systems are altered when PEG is introduced; this is particularly the case when the cargo is nucleic acids such as siRNA or DNA. In order for nucleic acids to be successful they must be delivered to the correct intracellular destination. PEG not only reduces the overall charge of the nanoparticles, which in turn lowers the cellular uptake, but also impairs disruption of the endosome. Therefore if PEGylation is to be used to enable EPR targeting, novel systems must be developed that can overcome the intracellular barriers to effective nucleic acid delivery.

4.2. Targeting ligands

One method of targeting is via the incorporation of targeting ligands that bind to cell-surface receptors. This approach is dependent upon possession of the knowledge of which receptor or combinations of receptors are hyperactivated on the cancer cell surface. One such example is the asialoglycoprotein receptor (ASGPr) which, although present on the surface of normal hepatocytes, is overexpressed in hepatocarcinoma cells. The ligand asialofetuin has been attached to a novel lipopolymeric nanoparticle to deliver the immunostimulatory IL-12 cytokine in the treatment of hepatocellular carcinoma. Following intratumoural administration of the targeted nanoparticles, the authors showed survival in 75% of mice treated with targeted nanoparticles compared to 38% in the non-targeted nanoparticles. This indicates that the presence of the ASGPr targeting ligand improves intracellular internalization via receptor-mediated endocytosis. Following systemic delivery of either nanoparticle type, luciferase expression in the liver and lungs was assessed. Luciferase expression was 10-fold higher in the livers of those mice that received targeted nanoparticles. However, there was also gene expression in the lung with no significant differences between targeted and non-targeted nanoparticles which indicates that further formulations may be necessary, and that evaluation of gene expression in all the organs is necessary to confirm appropriate targeting [123].

Another useful targeting ligand for cancer gene therapy is transferrin. Transferrin is overexpressed in many malignancies including breast, bladder and lung [124-126]. The differential expression of the transferrin receptor and its extracellular location make it an ideal target for systemic targeting. Systemic delivery of transferrin covalently linked to polyethylenimine has not only shown effective tumour targeting *in vivo*, but it can also shield the positive charge of the nanoparticles [127]. Studies by Kircheis showed that a lower molecular weight of PEI was less toxic and that the incorporation of 25% of the negatively charged lipophilic transferrin ligand gave an almost neutral zeta potential with a significant reduction in aggregation of erythrocytes. *In vivo* this translated into lower toxicity, one log greater gene ex-

pression than transferrin-free nanoparticles and a ‘shielding’ effect to bypass organs such as the lung and target the tumour [127].

Identification of overexpressed receptors can also lead to the development of tumour targeting peptides. Using phage display methods, the T7 peptide (HAIFYPRH) was identified and shown to specifically bind to the human transferrin receptor, with competitive studies indicating that T7 bound at a different site to transferrin [128]. This T7 peptide has recently been utilised for targeted co-delivery of the chemotherapy drug doxorubicin (DOX) together with the human TRAIL gene (Tumour necrosis factor Related Apoptosis-Inducing Ligand) to target gliomas which are known to overexpress the transferrin receptor [129]. DOX was conjugated with a pH linker (for endosomal release) to T7-modified dendrigraft poly-L-Lysine dendrimers which then condensed the pORF-hTRAIL DNA [129]. *In vitro* and *in vivo* evaluations revealed targeting via the transferrin receptor and accumulation of the nanoparticles in gliomas following systemic delivery with a synergistic effect. In addition, the targeted T7 nanoparticles induced much less off site toxicity while inducing a significant anti-tumour effect [129].

Other targeting ligands of note include the epidermal growth factor receptor that is upregulated in a number of solid tumours such as breast, prostate, colorectal, and ovarian [130]. Although some anti-cancer strategies are designed to prevent EGFR activation via small molecule inhibitors such as gefitinib or antibodies such as Cetuximab [131], an alternative is to exploit the differential expression of EGFR. Thiol functionalisation to attach the mouse EGF ligand to PEGylated branched PEI (25 kDa) has shown excellent *in vivo* targeting to hepatocellular carcinoma. Biodistribution studies illustrate quite clearly that there is significantly more expression of the luciferase gene in both Huh-7 and HepG2 HCC tumours compared to other organs following intravenous injection of the complexes [132]. The authors also found that any distribution of the DNA to the liver was exclusively in the Kupffer cells and not the epithelial cells, indicative of degradation.

The EGF-PEG-PEI system has also been used to selectively deliver synthetic double stranded RNA (poly IC) [133]. Typically dsRNA is found in virally infected cells and an associated response involves the induction of apoptosis and recruitment of inflammatory cytokines [134,135]. Delivery of poly IC with PEI₂₅-PEG-EGF killed up to 85% of EGFR-over-expressing glioblastoma multiforme cells *in vitro* via apoptosis after 1 hour [133]. This cytotoxic effect was significantly enhanced when the PEI was partially replaced with a PEI-Mellitin conjugate, which improved endosomal disruption, enabling greater delivery of the dsRNA to the cytoplasm. In addition, the intratumoural delivery of (poly IC) PEI-PEG-EGF+PEI-Mel complexes completely eradicated the intracranial tumours for more than 1 year [133]. Further studies have revealed that with further formulation of the delivery vehicle (Linear PEI-PEG 2 kDa-EGF), systemic delivery of poly IC can significantly reduce A431 tumour growth *in vivo* [136]. Similar to transferrin, polypeptides for EGFR have been isolated and used effectively in cancer gene therapy. Phage display revealed an 11 amino acid sequence (YHWY-GYTPQNVI) termed GE11 that has shown specificity to the EGFR after both *in vitro* and *in vivo* studies [137]. Furthermore, when the GE11 peptide was conjugated to PEI and compared with EGF-PEI, it was found that the latter enhanced mitogenic activity, which is clear-

ly undesirable in the cancer environment. The authors indicate that due to this lack of mitogenic activity, the GE11 ligand is safer *in vivo*, and delivery of the luciferase gene intravenously revealed an 18-fold increase in luciferase expression in human hepatoma SMMC-7721 tumours compared to non-targeted PEI [137].

The fibronectin attachment protein of mycobacterium has also been utilised as a targeting ligand to the fibronectin molecule on epithelial cell membranes [138]. The Fab receptor was conjugated to chitosan-DNA nanoparticles and delivered via an air jet nebuliser to enhance gene expression in the lung epithelium. Again using the luciferase reporter gene, studies revealed that there was a 16-fold increase in gene expression over the non-targeted chitosan nanoparticles [138]. Another example of exploitation of differential expression is in glioma brain capillary endothelial cells that have an upregulation of the lipoprotein receptor-related protein-1. The angiopep-2 peptide ligand (TFFYGGSRGKRNNFKTEEY) has been successfully conjugated to a polyamidoaminodendrimer (PAMAM) with a PEG spacer and studies showed that cellular uptake of the nanoparticles was targeting ligand dose-dependent, and that targeting to the brain was achieved following intravenous delivery [139]. The angiopep targeting system has also been utilised to achieve a therapeutic efficacy in the delivery of PAMAM-PEG-Angiopep/pORF-TRAIL to glial tumours [140]. The administration of these modified nanoparticles yielded an average survival time of 69 days compared to 30 days in the parental PAMAM-PEG/pORF-TRAIL nanoparticle-receiving mice [140].

There are of course numerous examples of systemic targeted delivery employing such ligands. The message that is apparent from all of these studies is that if there is enough information on the expression of a certain receptor, then the incorporation of its targeting ligand into cationic non-viral systems can significantly enhance tumour-targeted accumulation of the nucleic acid.

4.3. Affibody targeting

Affibodies are small stable alpha helical proteins that lack disulphide bonds, have a low molecular weight and are essentially designed to mimic the action of antibodies. An original affibody protein scaffold is used as a template from which combinatorial phage libraries can be generated and subsequently ligand-specific affibodies can be selected from using phage display technology. Such protein scaffolds have been generated from bacterial surface receptors such as the IgG binding domains of staphylococcal protein A (SPA). The 58 amino acid Z domain from staphylococcal protein A (SPA) is one such scaffold that has been used as a template for ligand specific affibodies [141,142].

With respect to cancer targeting, high affinity affibodies have been generated for Human Epidermal Growth Factor Receptor 2 [143], Epidermal Growth Factor Receptor [144], Insulin-like Growth Factor-1 Receptor [145] and Platelet Derived Growth Factor Receptor β [146]. Using radio-labelling, all of the affibodies have been shown to accumulate in tumours *in vivo* with an impressive level of specificity following systemic delivery. The affinity of the affibodies is an important factor and ideally should be in the nanomolar range for effective targeting. For example, the affinity levels of affibody $Z_{\text{HER2.4}}$ are 50 nmol/L [147] whereas using a one step affinity maturation process, Orlova and colleagues were able to generate the $Z_{\text{HER2.342}}$ affibody

which has an affinity level of 22 pmol/L [143]. The increased affinity translated into a 4-fold increase in tumour uptake of $Z_{\text{HER2:342}}$ four hours post-injection with clear contrast in imaging and stability at least up to 24 h post-injection. With respect to the first generation EGFR affibodies, the affinity was in the 150 nM range [148] which is sub optimal for effective systemic targeting. A similar one step maturation procedure for the EGFR affibody showed that affinity could be significantly improved whereby Z_{EGFR1907} had a K_d of 5.4 nM. Furthermore, there was significant uptake of the indium-111-labeled affibody Z_{EGFR1907} in A431 tumours and EGFR-expressing organs *in vivo* compared to the non-EGFR-affibody Z_{taq} [144].

Translational applications of these 2nd generation affibodies to date include therapeutic tools for diagnostic and imaging purposes to aid in the identification of molecular drug targets and for the stratification of cancer patient populations. However these highly stable, selective proteins are undoubtedly going to have a huge role in the advancement of targeted non-viral systems in cancer gene therapy. Recently a peptide chimera was designed that consisted of the cell penetrating peptide TAT (T), the DNA condensing motif Mu and the HER2 affibody (AF). The position of the AF was critical to ensure targeting functionality given that the affibody must be able to fold properly. Prior to synthesis, ITASSER software was employed to predict functionality based upon peptide design with a linker between TAT-MU and AF that was helical and should therefore ensure stability of the domains [149]. Studies with the purified recombinant TAT-Mu-AF showed that this ternary complex could condense DNA, confer protection from degradation by DNase I and offer stability in serum [150]. Using GFP DNA complexed at a 1:8:2 ratio, there was little uptake of the complexes in the HER2-null MDA-MB-231 cell line and green fluorescence in the HER2-expressing MDA-MB-453, SK-OV-3 and SK-BR-3 cell lines that was proportional to HER2 receptor density. Furthermore, the complexes were non-toxic and functional when injected intra-tumourally into the HER2 positive MDA-MB-453 breast tumours *in vivo* [150]. Unfortunately these ternary complexes as yet have not been administered intravenously, which is the ultimate test of functionality, given the range of extracellular barriers previously discussed. Nevertheless, this ternary peptide system holds a lot of promise in the next generation of targeted peptide/protein delivery systems for non-viral gene therapy.

PEGylated liposomes have also been synthesised conjugated to the $Z_{\text{EGFR:1907}}$ affibody with a cysteine residue at the C-terminus to form sterically stabilised affibody liposomes (SAL) [151]. Although the SAL system was loaded with the drug mitoxantrone (MTO), other macromolecules such as nucleic acids could be applied to this system. The MTO-SAL nanoparticles were tested for cytotoxicity on EGFR-expressing A431 and MDA-MB-468 cell lines with MCF-7 as a negative control. Results indicated that the MTO-SAL nanoparticles had no effect on the viability of the EGFR-negative MCF-7 cell line (IC₅₀ value > 100 μM , compared with an IC₅₀ of 18 μM for MTO alone), while the EGFR-expressing A431 (2.8 μM) and MDA-MB-468 (6.8 μM) cell lines were as sensitive to MTO-SAL nanoparticles as they were to MTO only (IC₅₀ 1.3 μM and 3 μM respectively) [151]. Taken together, these data suggest that SAL specifically delivered its MTO payload to the EGFR-expressing cells, and that EGFR-null cells were protected from MTO-induced cytotoxicity by the SAL vehicle. These studies illustrate that cysteine-modified affibodies can be targeting ligands on liposomal de-

livery vehicles. Furthermore, by ensuring that receptor-mediated endocytosis occurs via the affibodies, a protective effect is conferred on non-expressing receptor tissue which is highly attractive for the delivery of cytotoxic nucleic acids.

Another example of the use of the Z_{HER2:342} affibody [143] is in a multifunctional biopolymer system that comprises several discrete functions [152]. This system consists of a fusogenic peptide (FP) sequence H5WYG [153], a DNA-condensing and endosomolytic domain (DCE) with repeating sequences of arginine and histidine, a M9 nuclear localization domain (NLS) [154] and a C-terminal Z_{HER2:342} affibody [143]. What is particularly striking about this delivery system is that the authors have designed it taking into account all of the intracellular barriers, and with the use of discrete motifs, have attempted to overcome each hurdle to successful gene delivery. Engineered within this delivery system is also cathepsin D enzyme substrate (CS) to enable cleavage of the targeting motif from the rest of the vector in late endosomes [152]. The DNA sequence for FP-(DCE)3-NLS-CS-TM was cloned into an inducible expression system and the recombinant biopolymer was expressed and extracted using affinity and size exclusion chromatography [152]. The functionality of each discrete motif was proven and competitive inhibitor binding and transfection studies clearly indicated that the affibody ensured receptor-mediated endocytosis *in vitro* [152]. Transfection efficiency of 21% was achieved in the SKOV-3 HER2-expressing cell line, while efficiencies of only 0.1 and 2% were achieved in the non-expressing PC-3 and MDA-MB-231 prostate and breast cancer cell lines, respectively. *In vivo* delivery and evaluation of the immune response are critical for the future development of such smart biopolymer systems. Nevertheless, this study illustrates that high affinity affibodies can be functional in recombinant delivery vectors, thus enabling receptor targeting to occur.

4.4. Transcriptional targeting

Of course it may not be necessary to have a targeted delivery system to achieve expression of a desired gene in a particular tissue. Many tumours have a differential expression of a particular transcription factor that can be exploited and used to restrict gene expression to a particular site. Several promoters that are either tissue- or tumour-specific have been developed that can circumvent the issues surrounding targeting delivery systems. For example the differential expression in telomerase activity between tumour and normal tissue together with the identification of the minimal components necessary for telomerase activity has enabled the use of the human telomerase reverse transcriptase and the template containing telomerase (hTERT and hTER) promoters to control gene expression. Studies by Dufès et al showed that systemic delivery of polypropylenimendendrimers complexed with a TNF α -expressing plasmid under the control of hTER and hTERT gave regression of solid carcinomas in xenografts with 100% survival with no obvious signs of toxicity [155]. The hTERT promoter has also been used in a dual reporter system with the human alpha fetoprotein (hAFP) promoter to drive expression of MicroRNA-26a (MiR-26a), a known tumour suppressor downregulated in hepatocellular carcinoma (HCC) [156]. The dual promoter system significantly increased MiR-26a expression and reduced viability *in vitro* and *in vivo* compared to single promoter or constitutively driven MiR-26a constructs in the HCC cell lines [156].

Insulin-like Growth Factor 2 (IGF2) is involved in cellular proliferation and differentiation, but is also overexpressed in a variety of tumours such as bladder carcinoma [157]. IGF2 has a total of four promoters with P3 and P4 promoters responsible for IGF2 expression during foetal and tumour development [158]. P3 and P4 have been utilized to drive expression of the cytotoxic Diphtheria Toxin A gene both as a single promoter system and a dual promoter construct termed P4-DTA-P3-DTA [159]. Part of the rationale for this was related to the differential activation of both P3 and P4 regulatory sequences in human tumours, so a dual system would ensure induction of DTA in a larger population of tumours. Using PEI as the delivery vehicle, bladder carcinoma studies have revealed that P4-DTA-P3-DTA was superior *in vitro* and *in vivo* in both heterotopic and orthotopic bladder tumour models [159]. Similar studies have also been performed in glioma models utilising the cancer-specific H19 promoter in tandem with the P4-IGF2 promoter to selectively control DTA expression [160]. These dual systems have to-date focused on accessible tumours where intratumoural injection would suffice, but only systemic delivery of such systems will fully validate the transcriptional control afforded by these promoters.

Many cancers have the propensity to metastasize to bone, and such tumours acquire osteomimetic characteristics in order to adapt and thrive in the local bone environment. Disseminated bone deposits are resistant to conventional therapies and are particularly difficult to target. Osteocalcin is the most abundant noncollagenous bone matrix protein and is involved in the regulation of bone formation and resorption [161-163]. Osteocalcin is also overexpressed in a range of cancers including ovarian, lung, brain, breast and prostate [164-166]. The transcription factor largely responsible for activating the osteocalcin promoter is the master transcription factor RUNX2. RUNX2 is also highly expressed in tumours that metastasize to bone, and therefore widespread activation of the human osteocalcin (hOC) promoter should be achieved, regardless of the heterogeneous tumour microenvironment. The hOC promoter has been utilized to drive inducible nitric oxide synthase (iNOS) expression [167,168]. Commercially available liposomes were used as a delivery vehicle for the hOC-iNOS construct. This resulted in exquisite specificity for androgen-independent prostate cancer cells *in vitro*, coupled with cytotoxicity comparable to that of constitutively expressed iNOS. *In vivo* data also confirmed the potency of hOC-iNOS gene therapy in a mouse xenograft model of human prostate (PC-3) cancer. Multiple intra-tumoural injections slowed tumour growth dramatically and led to some complete responses. On average, tumour growth was delayed by 59 days compared to vector only controls. This data from these studies supports the premise that tumour-specific promoters can effectively drive iNOS monotherapy giving long term tumour control. Future work within this group is now focused on systemic delivery of hOC-iNOS gene therapy. The hOC promoter has also been delivered systemically to control expression of TK in a replication-defective adenovirus (Ad-hOC-TK) and early viral genes in a replication competent adenovirus (Ad-hOC-E1) [169]. The authors found that vitamins C and D₃ significantly increased the activity of the hOC promoter and that triple therapy with Ad-hOC-E1, vitamin D₃ and vitamin C resulted in complete regression in 38% of renal cell carcinomas *in vivo* following a single intravenous injection [169].

Targeting to a desired tissue is quite often the stumbling block to systemic cancer gene therapy. For the delivery of DNA, targeting can be achieved through the use of promoter sequences. The success of this method of targeting is reliant upon prior knowledge of a difference in transcription factor expression between the target and normal tissue. A delivery system could therefore be designed to condense the DNA, traverse cell membranes, disrupt endosomes and actively transport the payload to the nucleus without the added biophysical complications of having an external targeting motif. Such a delivery system would in theory deliver the DNA to all tissue, but the DNA would only be transcribed and translated where the desired transcription factor is present, namely the target tissue.

5. Multifunctional delivery

An understanding of the key biological barriers is critical to the success of a multifunctional delivery vehicle. Perhaps one of the most multifaceted delivery vehicles is the Multifunctional Envelope-type Nano Device MEND system [170]. The authors describe this as a 'programmed packaging' system whereby each part of the system is designed to carry out a specific function in a time-controlled manner. In this system the nucleic acid is condensed with a cationic polymer, wrapped in a lipid envelope which is then functionalised with PEG or other targeting ligands [170]. It is quite clear that PEGylated MEND did have a longer circulation time and was not rapidly cleared from the liver. These are ideal extracellular delivery characteristics, but unfortunately this translated into a much lower gene expression. Therefore circumvention of the 'PEG dilemma' could be achieved via the attachment of targeting ligands to receptors that are known to be over-expressed on tumour cells coupled with the attachment of a cleavable PEG that exploits either intracellular or tumour-specific characteristics. Figure 2 contains a representation of a MEND that highlights some of the nucleic acids that have been delivered and functionalisation strategies that have been used.

The avoidance of an immune response to non-viral strategies can also be greatly improved by use of MENDs as delivery devices. Delivery of pDNA to mice by a MEND resulted in differential expression of almost 1600 genes; PEGylation of the MEND reversed the altered expression of many of these genes. Gene Ontology analysis revealed that in general, the up-regulated genes were associated with "immune response," "response to biotic stimulus," "defence response," and related processes. The expression of IL-6, but not IFN α (commonly activated cytokines, as discussed above), were lower in the PEGylated MEND group compared with the non-PEGylated [171]. PEGylation has been shown to limit endosomal escape of gene delivery complexes [172]; inclusion of GALA in the MEND facilitated endosomal escape, and diminished the previously elevated IFN α levels [171]. A MEND functionalised with a PEG-peptide-DOPE conjugate (PPD) was stable in the systemic circulation after intravenous delivery (thereby benefiting from PEG's stabilising characteristic), while it also potentially delivered its pDNA cargo to HT1080 fibrosarcoma cells (MMP-rich), but not to HEK293 human embryonic kidney cells (MMP-deficient), thereby avoiding PEG's limiting characteristic [173]. Cleavage of PEG by MMPs facilitates the targeting of tumour tissues which are high in MMPs.

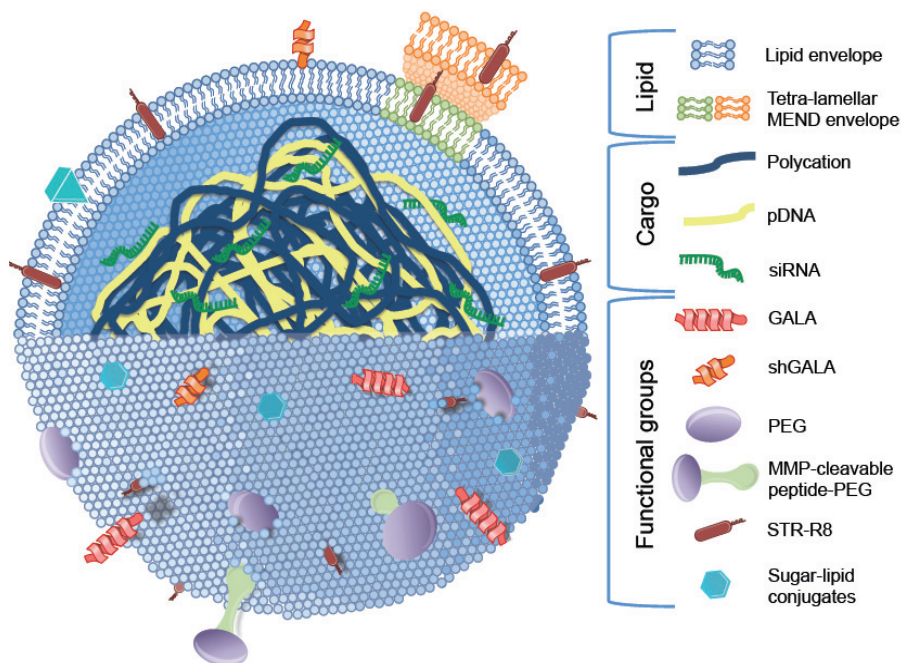


Figure 2. Simplified amalgamation of multifunctional envelope-type nano devices (MENDs) that have been employed for non-viral gene therapy development. pDNA cargoes encoding proteins such as luciferase [173-175] and GFP [176] have been delivered, as well as siRNA targeting luciferase [177,178] and ACTB [179]. MEND polycations are generally PLL [174,178] or protamine [173,175,176]. Lipid envelopes usually comprise DOTAP, DOPE and cholesterol [177,179,180], but can also include CHEMS [174,178]. Tetra-lamellar MEND envelopes comprise DOPE/cholesterol inner and DOPE/phosphatidic acid outer layers [176]. Functionalisation of MENDs with GALA/short GALA [179], STR-R8 [174,176,178], PEG and MMP-cleavable PEG [170,173,177] and sugar-lipid conjugates [175] have all been reported. Based on [181].

The fundamental limitation associated with non-viral gene therapies is their low transfection ability, compared with viral systems [1]. pDNA condensed using poly-L-lysine and incorporated into a MEND that comprised DOPE, cholesterylhemisuccinate and an octa-arginine (R8) peptide (DOPE/CHEMS/STR-R8) transfected HeLa and A549 cells as efficiently as an adenovirus vector. Moreover, the parity of transfection efficiency was achieved without negatively impacting cell viability, as was the case with adenovirus and Lipofectamine™, and evoked its therapeutic benefit following transdermal delivery in mice [181]. The R8 peptide has been used similarly to deliver proteins directly to cells [182].

A tetra-lamellar MEND (T-MEND) was nano-engineered that envelops the cationically-condensed pDNA in distinct functional layers to target the distinct barrier membranes faced by a nanoparticle. The pDNA-containing core was wrapped in a nucleus-fusogenic lipid membrane, which was in turn wrapped in an endosome-fusogenic lipid membrane that was

modified with a high density of octa-arginine. Upon endocytosis into the cell, the T-MEND's outermost membrane fuses with the endosomal membrane, releasing the nucleus-fusogenic lipid membrane-bound pDNA core from the endosome into the cytoplasm. The nuclear membrane is then overcome by fusion of the inner nucleus-fusogenic lipid membrane with the nuclear membrane, facilitating transport of the pDNA core into the nucleus. Despite the complexity of the particles, the fully-formed T-MEND produced particles of 163 nm diameter, and zeta potential of 54.5mV. Unsurprisingly, the T-MEND facilitated impressive pLuciferase delivery *in vitro* [176]. This exciting T-MEND was further functionalised by addition of fusogenic KALA to the outer and inner membranes, which improved transfection 20-fold [183]. To the authors' knowledge, systemic delivery of multi-layered MENDs is yet to be reported. Caution must be advised, as promising *in vitro* findings do not always translate into impressive *in vivo* developments.

6. Conclusion

It is apparent that the field of non-viral gene delivery is making significant progress in the quest for the ideal gene delivery vehicle. What is also evident is that the most successful systems are designed to overcome many biological barriers and as a consequence the traditional single function systems are now rendered obsolete. Viruses are nature's perfect delivery vehicle and provide the inspiration to many non-viral gene therapy researchers in the design of state of the art multi-faceted vehicles. Through a greater understanding and appreciation of the biological barriers to systemic gene delivery, non-viral gene therapy researchers are on the cusp of creating a variety of highly efficient vehicles that will revolutionise cancer gene therapy.

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Gene Therapy Based on Fragment C of Tetanus Toxin in ALS: A Promising Neuroprotective Strategy for the Bench to the Bedside Approach

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Additional information is available at the end of the chapter

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1. Introduction

Neurodegenerative diseases cover a wide range of neurogenetic disorders including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD), Huntington's disease (HD), the spinocerebellar ataxias, inherited prion diseases, the inherited neuropathies, and muscular dystrophies among others.

In particular, ALS belongs to the group of motor neuron diseases, involving the loss of cortex, brainstem, and spinal cord motor neurons that result in muscle paralysis [1]. Motor neurons, which are localized in the brain, brainstem and spinal cord, behave as a crucial links between the nervous system and the voluntary muscles of the body, as they let synaptic signals travel from upper motor neurons in the brain to lower motor neurons in the spinal cord and finally to muscles. In accordance with the revised El Escorial criteria [2], both the upper motor neurons and the lower motor neurons degenerate or die in ALS, and as a consequence the communication between neuron and muscle is lost, prompting the progressive muscle weakening and the appearance of fasciculations. In the later stages of the disease, patients become paralyzed although the disease usually does not impair a person's mind or intelligence.

Nowadays, the cause of ALS and its early manifestations still remain to be elucidated. The pathophysiological mechanisms that prompt the neurodegenerative process in both familial (FALS) and sporadic (SALS) ALS are unknown. However, there is growing evidence that the pathogenic process involved in ALS are multifactorial and include oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, axonal transport systems and dysfunction of glial cells, yielding the damage of critical proteins and organelles in the motor neuron triggering the neurodegeneration [3]. Due to the fact that FALS and SALS share clinical and

pathological signs, the understanding of the pathophysiological process in FALS would provide a better understanding of the neurodegenerative mechanisms in SALS.

FALS follows a predominantly autosomal dominant pattern, while in SALS genetic factors that take place sporadically contribute to its pathogenesis. The majority of ALS cases are sporadic and 5-10% of cases correspond to FALS. Although the ages of onset of FALS, which follow a normal Gaussian distribution, correspond to a decade earlier than for SALS cases which have an age dependent incidence, males and females are affected equally in FALS [4].

The most significant candidate genes for SALS include *VEGF* (vascular endothelial growth factor), *angiogenin* (*ALS9*), *paraoxonase*, *neurofilaments*, *peripherin* and *SMN* (spinal muscular atrophy). Although *ALS9*, *paraoxonase*, *neurofilaments*, *peripherin* and *SMN* mutations have been found in ALS patients, except for *VEGF* mutations, these genes may play a small role in the pathogenesis of ALS and previous studies are conflicting [5].

Regarding FALS candidate genes, the mutations in the copper/zinc superoxide-dismutase-1 gene (*SOD1*), Tar DNA-binding protein gene (*TARDBP*) and in the most recent discovered DNA/RNA-binding protein called *FUS* (fused in sarcoma) or *TLS* (translocation in liposarcoma) produce the typical adult onset ALS phenotype. Other candidate genes that have been described in genome association studies of FALS include *dynactin*, *senataxin* (*ALS4*) and *VAPB* (*ALS8*) (*VAMP/synaptobrevin-associated membrane protein B* gene) [5,6].

The pathophysiology of *SOD1* mutations is probably the most studied one. Many hypotheses have been suggested and reinforced in transgenic mouse models that overexpress the mutated *SOD1* gene and therefore develop an ALS-like syndrome. Among the proposed mechanisms that support these hypotheses are the toxic gain of function of the mutated *SOD1* enzyme, which mainly increases the production of hydroxyl and free radicals, yielding improper binding metal properties, oxidative stress and inflammation induced by upregulation of proinflammatory cytokines [7,8]. Alternative hypothesis also suggested a conformational instability and misfolding of the *SOD1* peptide, forming intracellular aggregates which have been reported in motor neuron and glial cells [9].

Neurotrophic factors have been initially identified as potential therapeutic agents in the treatment of ALS, opening the door to a new tool for the treatment of motor neuron diseases [10]. Based on previous studies ciliary and glial derived neurotrophic factors, insulin-like growth factor (IGF-1) and erythropoietin improved motor behaviour and reduce motor neuron loss, astrocyte and microglia activation in preclinical animal models [11], albeit clinical trials in ALS patients showed lack of therapeutic efficacy [12].

The failure of standard treatments in ALS could rely on the inappropriate route of administration and/or the poor bioavailability of molecules to the target cell [13]. The subcutaneous and intrathecal delivery of neurotrophic factors can cause adverse side effects such as weight loss, fever, cough, fatigue and behavioral changes [14], whereas viral gene therapy based on the use of an adeno-associated virus or lentivirus vectors is more efficient than the neurotrophic factor delivery but can induce several inherent hazards [15].

An alternative strategy that effectively reaches motor neurons, can exert neuroprotective properties and does not show such adverse side effects implies the use of the nontoxic fragment C

(TTC) of tetanus toxin. Tetanus toxin is a neurotoxin produced by *Clostridium tetani*, an anaerobic bacterium whose spores are commonly found in soil and animal waste. This toxin affects the nervous system and causes generalized muscle contractions, called titanic spasms [16, 17].

Tetanus toxin is a single peptide of approximately 150 kDa, which consists of 1315 amino-acid residues. The toxin forms a two-chain activated molecule composed of a heavy chain (HC) and a light chain (LC) linked by a disulfide bond. The catalytic domain of the toxin resides in the LC, while the translocation and receptor-binding domains are present in HC [18–21] (Figure 1). Tetanus and botulinum toxins are zinc metalloproteases that cleave SNARE (soluble NSF attachment receptor) proteins, which interfere with the fusion of synaptic vesicles to the plasma membrane and ultimately blocks neurotransmitter release in nerve cells [22].

The nature of the action of tetanus toxin has been widely described in different animal models [23–28], exploring its effect not only in the spinal cord but also in the cerebral cortex [29]. One of the unique characteristics of tetanus toxin is that it can be transported retrogradely to the central nervous system and shows remarkable affinity and specificity to neuronal terminals. The ganglioside-recognition domain in the C-terminal region of HC allows the toxin to be internalized into the neuron at the neuromuscular junction where it enters the axonal retrograde transport pathway and is subsequently transported to the neuronal soma in the CNS [30,31]. Once the toxin reaches the cytoplasm, it specifically cleaves neuronal proteins integral to vesicular trafficking and neurotransmitter release. In particular, the synaptic vesicle protein synaptobrevin (VAMP) is the target of tetanus toxin. This protein belongs to a family of proteins that facilitate exocytosis in neurons known as SNARE proteins. The other members of this family are syntaxin and SNAP-25, which are the main molecular targets of botulinum toxin. SNARE proteins are formed by coiled-coil interactions of the alpha-helices of its members, which is required for membrane fusion [32–35].

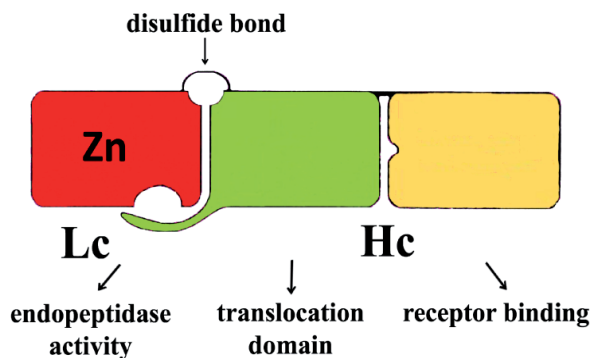


Figure 1. Diagram of the tetanus toxin molecule. The targeting and the translocation domains are located in the heavy-chain (HC), whereas the catalytic domain is located in the light-chain (LC) of the molecule. Its proteolytic activity is Zn^{2+} -dependent, and heavy-metal chelators generate inactive apo-neurotoxins. TTC is approximately 50 kDa and resides in the HC of the toxin. The ganglioside-recognition domain in TTC allows the toxin to be internalized into the neuron [35].

From the gene therapy point of view, the most interesting part of the toxin that must be outstanding is TTC. This fragment of the toxin is located in the HC of tetanus toxin molecule and it plays an important role in the neuronal internalization (Figure 1). In fact, TTC maintains transport properties of the native tetanus toxin without causing toxic effects, in such a way that in the absence of TTC, the toxin retains little ability to paralyze neuromuscular transmission [35,36].

The trans-synaptic transport of TTC was intensively studied in one of the best-characterized systems, the primary visual pathway [37, 38], confirming its capacity as a carrier once it was injected intramuscularly [39-41]. Furthermore, the possibility of constructing recombinant molecules with TTC has opened the door to an interesting research field, the discovery of neuro-anatomical tracers, whose main purpose is to map synaptic connections between neuronal cells.

One of the most well-known recombinant proteins that have been used for this purpose is the protein encoded by *lacZ*-TTC. This protein has been tested *in vitro* and *in vivo* to determine its activity in the hypoglossal system, and the detection of the labeled motor neurons was dependent on time post-injection [40-42]. Since neuronal integrity is crucial for TTC internalization, the transneuronal molecular pathway at neuromuscular junctions was intensively studied using this recombinant protein [43]. The protein was detected not only in the neuromuscular junction postsynaptic side but also the soma of the motor neuron, away from the active zones in large uncoated vesicles.

The advances in the understanding of these recombinant proteins have paved the way for new therapeutic approaches using TTC as a carrier of molecules to ameliorate the disease process of motor neuron diseases, neuropathies and pain. As an example, several proteins conjugated to TTC that have been used to study neuronal internalization *in vitro* and *in vivo* are horseradish peroxidase (HPRT), glucose oxidase (GO), green fluorescent protein (GFP), β -N-acetylhexosaminidase-A (HEXA), superoxide dismutase 1 (SOD1), survival motor neuron 1 (SMN1), cardiotrophin-1 (CT1), B-cell lymphoma-extra large (Bcl-xL), IGF-1, glial derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF) [17]. More recently, a novel multi-component nanoparticle system using polyethylene imine (PEI) has been evaluated to elicit the expression of BDNF in neuronal cell lines [44].

Apart from the carrier properties of TTC, the neuroprotective nature of TTC was one of the best kept properties to discover.

The neurotrophin family has been shown to regulate survival, development and functional aspects of neurons in the central and peripheral nervous systems through the activation of one or more of the three members of the receptor tyrosine kinases (TrkA, TrkB, and TrkC) in cooperation with p^{75NTR} [45-48]. Nerve growth factor (NGF) can bind to the TrkA receptor or a complex of TrkA and p^{75NTR} [45], BDNF and neurotrophin-4/5 can bind to TrkB, and neurotrophin-3 binds to TrkC. Interestingly, the retrograde pathway of TTC is shared by p^{75NTR} , TrkB and BDNF, which is strongly dependent on the activities of the small GTPases Rab5 and Rab7 [49], therefore TTC alone might have a neuroprotective role and therefore it can be a valuable non-viral therapeutic agent in ALS.

2. Neuroprotective nature of TTC

Many authors have suggested that the trans-synaptic transcytosis pathway used by tetanus toxin was most likely “designed” for the trafficking of trophic factors through a chain of connected neurons [50]. Furthermore, two trophic factors, GDNF and BDNF, have been reported to possess similar trans-synaptic transcytotic properties to those of tetanus toxin [51].

Tetanus toxin can induce an increase in serotonin synthesis in the central nervous system, suggesting that the toxin-affected serotonergic innervation in the perinatal rat brain can trigger the translocation of calcium phosphatidylserine-dependent protein kinase C (PKC) [52]. In particular, tetanus toxin is able to alter a component involving inositol phospholipid hydrolysis, which is associated with PKC activity translocation [53,54]. In addition to this translocation, an enhancement of the tyrosine phosphorylation of the tyrosine receptor TrkA, phospholipase C (PLC γ -1) and ERK-1/2 can be also observed [55]. Due to the fact that TTC can stimulate the PLC-mediated hydrolysis of phosphoinositides in rat brain neurons, TTC seems to modulate some signaling pathways involving the transport of serotonin [56].

Moreover, the activation of intracellular pathways related to the PLC γ -1 phosphorylation and activation of PKC isoforms and the kinases Akt (at Ser 473 and Thr 308) and ERK-1/2 (at Thr 202/Tyr 204) is induced by TTC in rat brain synaptosomes and cultured cortical neurons. This signal pathway activation is dependent on time and concentration, therefore TTC can exert neuroprotective effects, activating TrkA and TrkB receptors in a similar manner as do NGF and BDNF or neurotrophin-4/5 [57,58].

The neuroprotective role of TTC is also supported by the fact that it can also protect cerebellar granular cells against potassium deprivation-induced apoptotic death [59] and act as a neuroprotector in a model of 1-methyl-4-phenylpyridinium (MPP⁺)-triggered apoptosis, enhancing the survival pathways in rats with a dopaminergic lesion and improving different motor behaviors. Particularly, TTC is able to induce Ser 112 and Ser 136 BAD phosphorylation, activate the transcription factor NF- κ B, which prevents neuronal death, and induce a decrease in the release of cytochrome c and, consequently, a reduction in the activation of procaspase-3 and chromatin condensation [60,61].

More recently, the nature of TTC described by Longstreth and colleagues [62] and Larsen and colleagues [63], based on its stability to reach motor neurons specifically through the retrograde axonal transport system, has been reinforced as a potential neuroprotective agent in previous *in vivo* studies of gene and protein expression after injection of plasmid-DNA in transgenic SOD1^{G93A} mice, which carries the mutation G93A in human superoxide dismutase 1 (SOD1) [64]. These studies suggested that intramuscular naked-DNA TTC gene therapy administered into neurodegenerative mouse model delayed the onset of symptoms (by approximately 5 days), prolonged survival (by approximately 13 days) and improved the motor function activity in TTC-treated mice throughout disease progression, by increasing numbers of surviving motor neurons (Figure 2).

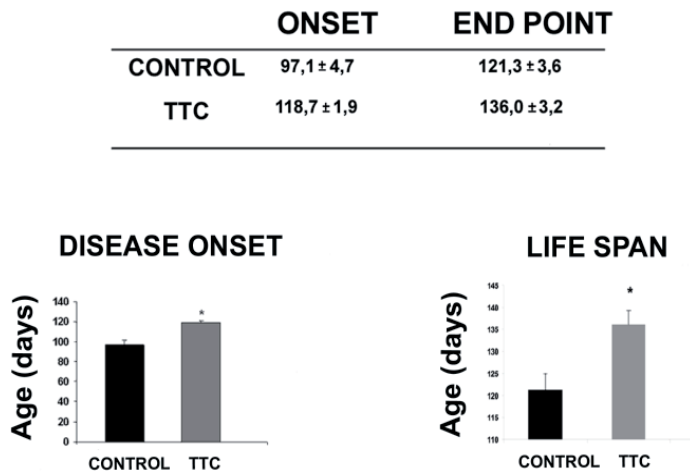


Figure 2. Functional and survival effect under TTC treatment. Intramuscular injection of TTC-encoding plasmid in SOD1^{G93A} mice (grey bars) delays significantly disease onset and mortality compared to the control group (*p<0,05, error bars indicate SEM) (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

Apart from functional and survival results obtained *in vivo* in transgenic SOD1^{G93A} mice, the electrophysiological studies showed that, from three to four months of age, TTC treatment played a partial protective effect as demonstrated by the lower decline in amplitudes of the M waves, improvement in motor behavioral tests, and increased survival of motor neurons in the TTC-treated animals’ lumbar spinal cord [64] (Figure 3).

Interestingly, TTC administration can also affect antiapoptotic pathways by means of calcium-related mechanisms [64]. The positive effects on motor neuron preservation, animal motor function, and survival were confirmed with studies of anti-apoptotic effects and survival signals in the spinal cords of treated animals. Transcriptional caspase-1 and caspase-3 levels were downregulated in the spinal cord of TTC-treated animals as well as significant variations in calcium-related gene expression were found [64]. Furthermore, a downregulation of the caspase-3 activation protein levels in the spinal cord of TTC-treated animals indicated that TTC might act through an anti-apoptotic pathway. Actually, Bax, Bcl2, phospho-Akt and phospho-ERK 1/2 protein expression levels in TTC-treated animals were statistically significant and close to those of wild-type animals, suggesting a decrease of apoptosis and a lower degree of motor neuron neurodegeneration due to TTC treatment [64].

Taking all these results obtained *in vitro* and *in vivo* as a whole, non-viral gene therapy treatment based on TTC could be a safe and promising neuroprotective strategy for neurodegenerative diseases, especially in ALS. However, the next question to be tackled is whether a recombinant molecule of TTC may have a synergistic effect and enhance the neuroprotective properties of TTC alone.

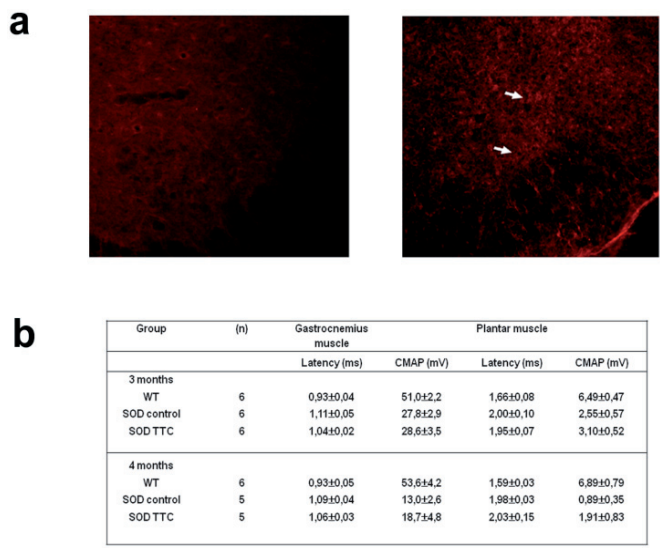


Figure 3. Motor neuron survival and neurophysiological study in gastrocnemius and plantar muscles in SOD1^{G93A} mice. (a) Presence of TTC in the grey matter of the ventral horn of (a) positive control (SOD1^{G93A} transgenic mice injected with empty plasmid) and (b) SOD1^{G93A}-TTC treated mice. Arrows point to some of the neurons positively stained for TTC. Bar = 200 μ m. (b) Electrophysiological study of compound muscle action potential (CMAP) in gastrocnemius and plantar muscles in wild-type mice (WT), control SOD1^{G93A} mice, and SOD1^{G93A} mice treated with naked DNA encoding for TTC. Values are the mean \pm SEM. CMAP, compound muscle action potential; n, number of mice. *P < 0.05 vs. WT group at the same age (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

3. Neuroprotective properties of recombinant molecules of TTC in a mouse model of ALS

It has been very well described the specificity of a trophic factor for motoneurons and precisely this specificity could be increased by genetically fusing it to TTC, while the trophic factor could contribute to enhance the benefits observed for TTC. Therefore the next inevitable approach is to test naked-DNA gene delivery to encode for a chimeric molecule, to study the potential synergistic effect.

As previously mentioned, BDNF belongs to the family of neurotrophins and binds specifically to TrkB receptors to activate the intracellular signaling pathways that promote neuronal survival and the differentiation of neurons. The neurotrophic effects of BDNF on motoneuronal degeneration have been widely studied *in vitro* and *in vivo* [66,67]. This neurotrophin has also been proposed as a potential therapeutic agent for the treatment of human ALS [68], although no successful results have been achieved. This failure in the clinical

application of BDNF may be due to the low efficacy of targeting the neurotrophic factor to motoneurons. Alternatively, TTC possesses a high affinity for motoneurons [40], and the fusion of BDNF to the TTC protein might increase its accessibility. A previous study reported that some neurotrophic factors, in particular BDNF, facilitate the internalization of TTC recombinant molecules in motor nerve terminals [69]. In addition, TTC and the recombinant protein BDNF-TTC can inhibit apoptosis in cultured neurons, with the quimeric molecule being more effective than TTC alone [70]. Interestingly, BDNF may cause a relocation of membrane domains containing TTC receptors by activating Trk receptors, thereby facilitating the neuronal internalization of TTC. This observation is supported by other authors who state that TTC activates intracellular pathways involving Trk receptors [58]. Therefore, the hypothesis of a synergistic positive effect based on the fusion of the mature form of BDNF genes to TTC in a mouse model of ALS needs to be pointed out for the bench to the bedside approach.

Similarly to the results observed in transgenic SOD1^{G93A} mice [64], an amelioration of the decline in hindlimb muscle innervation was observed in the animals that were injected with either naked DNA encoding TTC or naked DNA encoding the recombinant molecule TTC and BDNF (BDNF-TTC) [65] (Figures 4,5), in addition to a significant delay in the onset of symptoms and functional deficits (Figure 6), an improvement in the spinal motor neuron survival (Figure 7) (down-regulation of caspase-1 and caspase-3 levels and a significant phosphorylation of serine/threonine protein kinase Akt) (Figure 8) and a prolonged lifespan under both treatments [64,65].

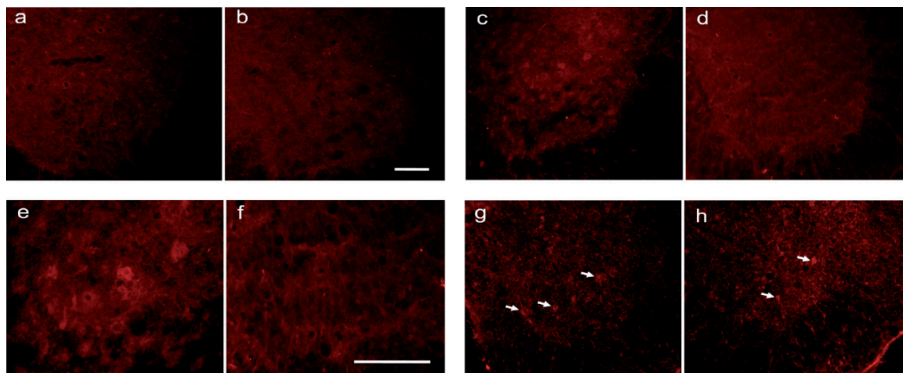


Figure 4. Motoneuronal preservation in transgenic SOD1^{G93A} mice under TTC, BDNF and BDNF-TTC treatments. Immunohistochemical labeling for BDNF expression in the grey matter of the ventral horn of (a) positive control (SOD1^{G93A} transgenic mice injected with empty plasmid), (b) SOD1^{G93A}-BDNF and (c) L2 and (d) L4 spinal segments of SOD1^{G93A}-BDNF-TTC mice. (e, f) Detail of BDNF immunolabeling of the sections shown in c and d, at higher magnification. Presence of TTC in the grey matter of the ventral horn of (g) SOD1^{G93A}-BDNF-TTC and (h) SOD1^{G93A}-TTC treated mice. Arrows point to some of the neurons positively stained for TTC. Bar = 200 μ m in a, b, c, d, g and h; bar = 100 μ m in e and f (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

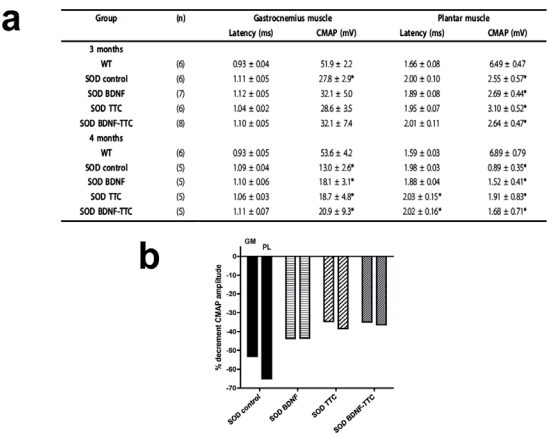


Figure 5. Neurophysiological study in gastrocnemius and plantar muscles. (a) Results of wild-type mice (WT), control SOD1^{G93A} mice, and SOD1^{G93A} mice treated with naked DNA encoding for BDNF, TTC, and BDNF-TTC are shown. Values are the mean ± SEM. CMAP, compound muscle action potential; n, number of mice. *p < 0.05 vs. WT group at the same age. (b) Histogram representation of the decrement in the amplitude of the compound muscle action potential. CMAP was compared at 4 months with respect to values at 3 months of age in SOD1^{G93A} mice, untreated and treated with naked DNA encoding for BDNF, TTC or BDNF-TTC. For each group, the left bar corresponds to the gastrocnemius muscle and the right bar to the plantar muscle (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

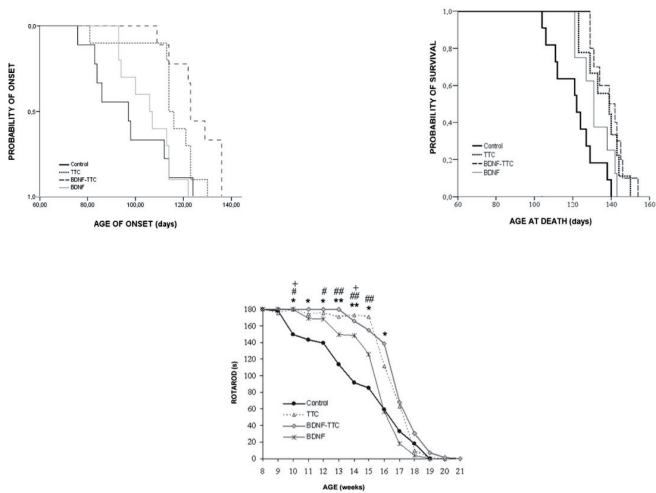


Figure 6. Improvement in disease clinical outcomes in transgenic SOD1^{G93A} mice under TTC, BDNF and BDNF-TTC treatments. Cumulative probability of the onset of disease symptoms (hanging-wire test) and survival in SOD1^{G93A} mice injected

at 60 days of age with TTC, BDNF-TTC, BDNF or empty (positive control) plasmids. Strength and motor function were tested using the rotarod at 15 rpm. Mice were given up to 180 s for the test performance and the time at which mice fell was recorded (*, #, +, $P < 0.05$; **, ##, $P < 0.01$; error bars indicate SEM); * for BDNF-TTC vs. positive control comparisons; # for TTC vs. control comparisons; + for BDNF vs. positive control comparisons (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

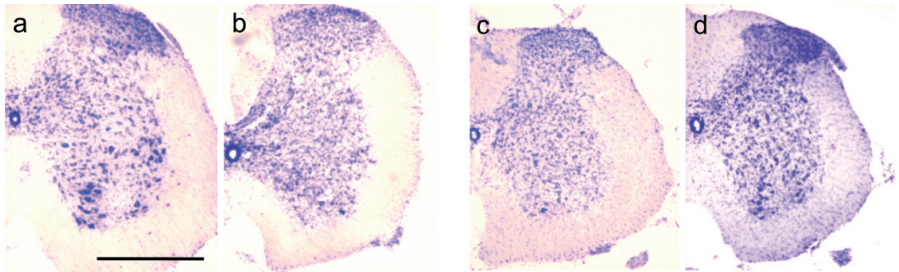


Figure 7. Spinal motor neuron survival of transgenic SOD1^{G93A} mice under TTC, BDNF and BDNF-TTC treatments. Representative micrographs showing cross-sections of lumbar spinal cords stained with cresyl violet from wild-type, (a) SOD1^{G93A} control (positive control), (b) BDNF-treated, (c) and BDNF-TTC-treated, (d) mice at 16 weeks of age. Bar = 500 μm (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

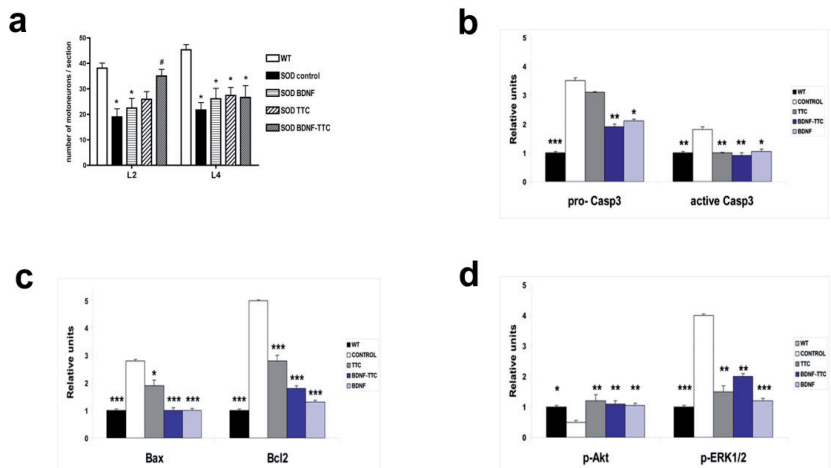


Figure 8. Apoptotic and survival pathways under TTC, BDNF and BDNF-TTC treatments. (a) Histogram representation of the average number of stained motoneurons per section in L2 and L4 spinal cord segments of wild-type littermates, control SOD1^{G93A} and treated mice ($n = 4-5$ mice per group). * $p < 0.05$ vs. wild type; # $p < 0.05$ vs. SOD^{G93A} control mice. (b) Fold-changes in the expression of pro-Casp3 and active Casp3 proteins, (c) Bax and Bcl2 proteins and (d) phosphorylated states of Akt and ERK1/2 proteins in spinal cord lysates of control SOD1^{G93A} animals (white) and ani-

mals treated with TTC (grey), BDNF-TTC (blue, BTTC) and BDNF (soft blue). Western blot quantities are shown as the ratios to b-tubulin and then related to age-matched wild-type (black) mice data (* $P < 0.05$ and ** $P < 0.01$ vs. control SOD1^{G93A} mice; *** $p < 0.001$; error bars indicate SEM) (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

Additionally, GDNF is another candidate neurotrophic factor for ALS therapy. This factor has been described to show potent trophic effects on proliferation, differentiation and survival of motor neurons *in vitro* and *in vivo* [63,71-76]. Furthermore, after the retrograde transport of GDNF to the cell bodies, a fraction of this trophic factor avoided degradation and was sorted to dendrites [51], similar to the known movement of the TTC [39]. It was also suggested that the transsynaptic and transcytotic pathway used by GDNF was similar to that of TTC, but not identical, and that GDNF protein degradation was lower than that of TTC protein. Furthermore, the combination of TTC and GDNF has been evaluated in a neonatal rat axotomy model [63] and in the ALS mouse model [77]. The combination of TTC with insulin growth factor (IGF-1) has also been assayed in transgenic SOD1^{G93A} mice [78], although the effect of TTC alone has not been compared in any of these studies. When the effect of TTC was compared to the recombinant molecule *in vitro*, a significant increase in the survival capacity of neuronal cells was found [77]. However *in vivo*, no significant differences were observed, which is probably due to the possibility that the recombinant molecule might follow a GDNF route and not the TTC route under axotomy conditions [63].

When focusing the study *in vivo* in a mouse model of ALS, the recombinant molecule TTC and GDNF (GDNF-TTC), GDNF and TTC treatments prompted a delay in disease onset, an improvement in motor function and a longer lifespan in transgenic SOD1^{G93A} mice, comparing to empty-plasmid injected control mice [79] (Figure 9).

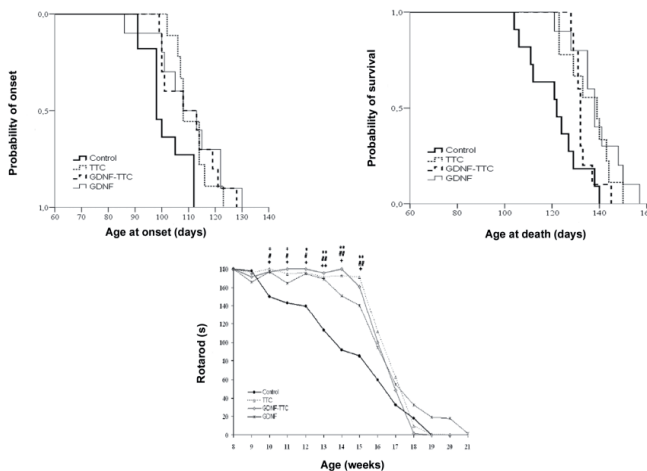


Figure 9. Improvement in disease clinical outcomes in transgenic SOD1^{G93A} mice under TTC, GDNF and GDNF-TTC treatments. Cumulative probability of the onset of disease symptoms (hanging-wire test) and survival in SOD1^{G93A} mice injected at 60 days of age with TTC, GDNF-TTC, GDNF or empty (positive control) plasmids. Strength and motor

function were tested using the rotarod at 15 rpm. Mice were given up to 180 s for the test performance and the time at which mice fell was recorded (*, #, +, $P < 0.05$; **, ##, $P < 0.01$; error bars indicate SEM); *GDNF-TTC vs. control comparisons; # TTC vs. control comparisons; + GDNF vs. control comparisons (*, #, +, $P < 0.05$; **, ##, $P < 0.01$; error bars indicate SEM) (Reprinted from Restor. Neurol. Neurosci, 30, Moreno-Igoa M, Calvo AC, Ciriza J. et al. Non-viral gene delivery of the GDNF, either alone or fused to the C-fragment of tetanus toxin protein, prolongs survival in a mouse ALS model, p. 69-80, Copyright (2012), [79] with permission from IOS Press).

Moreover, the recombinant molecule GDNF-TTC and full-length GDNF inhibited apoptotic pathways in spinal cords of SOD1^{G93A} mice by reducing the activation of caspase-3, as well as Bax and Bcl2 protein levels reached a profile expression similar than the one observed in wild type mice, highlighting the fact that treated mice biochemically resemble non-transgenic mice (Figure 10). In addition, all treatment molecules activated the PI3K survival pathway by phosphorylating Akt and ERK1/2, resembling again the wild type levels [79] (Figure 10).

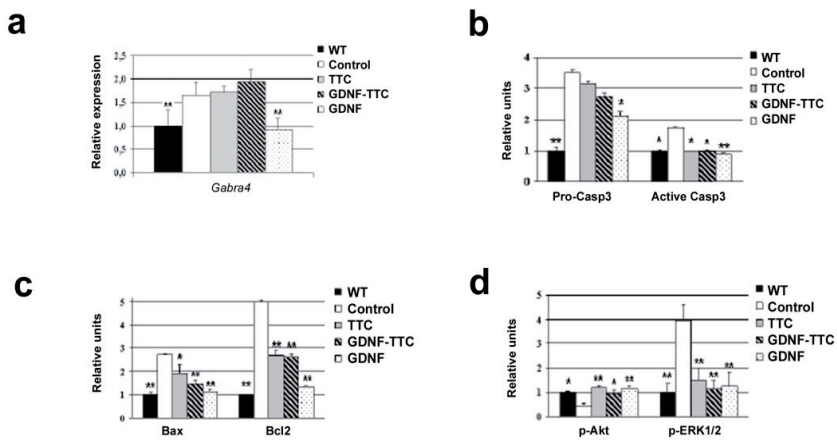


Figure 10. Apoptotic and survival pathways under TTC, GDNF and GDNF-TTC treatments. (a) Fold-changes in the expression of GABA(A) receptor subunit-4 (*Gabra4*) mRNA levels in total spinal cord of wild type, control transgenic mice and treated transgenic mice ($n=5$ per group). (* $P < 0.05$, ** $P < 0.01$; error bars indicate SEM). Fold changes in the expression of (b) pro-Casp3 and active Casp3 proteins, (c) Bax and Bcl2 proteins, and (d) phosphorylated states of Akt and ERK1/2 proteins. Western blots from spinal cord lysates of control animals (white) and treated with TTC (gray), GDNFTTC (hatched-columns) and GDNF (dotted-columns). Western blot quantities are shown as the ratio to β -tubulin and then related to age-matched wild type (black) mice data (Reprinted from Restor. Neurol. Neurosci, 30, Moreno-Igoa M, Calvo AC, Ciriza J. et al. Non-viral gene delivery of the GDNF, either alone or fused to the C-fragment of tetanus toxin protein, prolongs survival in a mouse ALS model, p. 69-80, Copyright (2012), [79] with permission from IOS Press).

Summarizing, albeit a significant improvement in behavioral assays together with an activation of anti-apoptotic and survival pathways under BDNF and GDNF treatments was observed in transgenic SOD1^{G93A} mice, no synergistic effect was found neither using the BDNF-TTC nor GDNF-TTC recombinant molecules. Interestingly, recombinant plasmids BDNF-TTC and GDNF-TTC were detected in skeletal muscle and the corresponding recombinant protein reached the spinal cord tissue of transgenic SOD1^{G93A} mice (Figure 11), reinforcing the carrier properties of TTC.

As a final point, the active state of the neurotrophic factors BDNF and GDNF in the recombinant molecule could suggest that either BDNF or GDNF could exert an autocrine and neuroprotective role together with TTC to a similar extent as TTC alone; however this effect could not be sufficient enough to prompt a synergistic effect. As a consequence, the recombinant molecules could mainly use the same pathway that mimics a neurotrophic secretion route, prompting survival signals in the spinal cord of transgenic SOD1^{G93A} mice [65,79]. Despite all these contributions to the understanding of the neuroprotective properties of recombinant molecules, it is undoubtedly that TTC has open the door to an alternative therapeutic strategy for more neurodegenerative diseases although its molecular pathways is not yet well characterized.

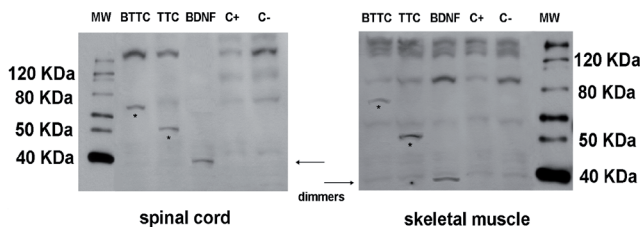


Figure 11. TTC and BDNF detection in skeletal muscle and spinal cord of ALS transgenic SOD1^{G93A} mice. Western blot detection of TTC in spinal cord and skeletal muscle tissues of wild-type (C-, negative control), SOD1^{G93A} transgenic mice injected with empty plasmid (C+, positive control), TTC- and BDNFTTC (BTTC)-treated mice. In TTC and BDNF-TTC treated groups, the detected band was approximately of 50 and ~ 70 KDa respectively (*), using both anti-TTC and anti-BDNF antibodies. In the BDNF group, the dimeric conformation, indicated by arrows, was observed at approximately 40 KDa (Reprinted from Orphanet J. Rare Dis., 6: 10, Calvo AC, Moreno-Igoa M, Mancuso R. et al. Lack of a synergistic effect of a non-viral ALS gene therapy based on BDNF and a TTC fusion molecule, Copyright (2011), [65] with permission from BioMed Central).

4. Conclusions

At present, gene and stem cell therapies are holding the hope for an efficient treatment in ALS. Regarding gene therapy, the possibility of delivering therapeutic molecules to damaged tissues crossing the blood-brain barrier has made possible the study of viral (adenovirus, adeno-associated and lentivirus) and non-viral (fragment C of tetanus toxin) vectors, which are retrogradely transported to motor neurons, in preclinical animal models showing promising neuroprotective effects.

Although therapeutic strategies, which tend to stop or slow down the progression of ALS, are one of the main goals in this field of research, the new property of TTC has opened the door to new non-viral therapeutic strategies in this disease. The fact that TTC as well as the recombinant molecules BDNF-TTC and GDNF-TTC can be transported through motoneurons to induce a later onset of symptoms, improve motoneuron survival and extend the survival of SOD1^{G93A} mice support the fact that the naked DNA-mediated intramuscular

delivery of TTC and fusion molecules can promote neuroprotective effects in the SOD1^{G93A} murine model of ALS. The active states of BDNF and GDNF in the recombinant molecules also confirm that these neurotrophic factors could exert an autocrine and neuroprotective role together with TTC to a similar extent as TTC alone, but this effect was not sufficient to enhance the survival signals observed under TTC treatment alone.

Definitively, the neuroprotective role of fragment C has shed light on the understanding of the disease neurodegeneration processes and the study of this promising property of TTC can be extended to other neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and Spinal Muscular Atrophy (SMN). Essentially, a better understanding of these neurodegenerative diseases will facilitate the translation from animal model to patients to find a definitive therapeutic approach.

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Transposons for Non-Viral Gene Transfer

Sunandan Saha and Matthew H. Wilson

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52527>

1. Introduction

DNA based transposon vectors offer a mechanism for non-viral gene delivery into mammalian and human cells. These vectors work via a cut-and-paste mechanism whereby transposon DNA containing a transgene(s) of interest is integrated into chromosomal DNA by a transposase enzyme. The first DNA based transposon system which worked efficiently in human cells was *sleeping beauty*. This was followed a few years later by the use of the *piggy-Bac* transposon system in mammalian and human cells. The advantages of transposon vectors include lower cost, less innate immunogenicity, and the ability to easily co-deliver multiple genes when compared to viral vectors. However, when compared to viral vectors, non-viral transposon systems are limited by delivery to cells, they are possibly still immunogenic, and they can be less efficient depending on the cell type of interest. Nonetheless, transposons have shown promise in genetic modification of clinical grade cell types such as human T lymphocytes, induced pluripotent stem cells, and stem cells. Recently generated hyperactive transposon elements have improved gene delivery to levels similar to that obtained with viral vectors. In addition, current research is focused on manipulating transposon systems to achieve user-selected and site-directed genomic integration of transposon DNA cargo to improve safety and efficacy of transgene delivery. DNA based transposon systems represent a powerful tool for gene therapy and genome engineering applications.

2. Transposons as gene delivery systems

Transposons or mobile genetic elements were first described by Barbara McClintock as “jumping genes” responsible for mosaicism in maize [1]. Transposons are found in the genome of all eukaryotes and in humans at least 45% of the genome is derived from such ele-

ments [2]. Transposons active in eukaryotes can work either by a “copy and paste” (Class I) or “cut and paste” (Class II) mechanism (Figure 1).

In the “copy and paste” mechanism, the transposon first makes a copy of itself via an RNA intermediate (hence also known as retrotransposons). Class II DNA-transposons work by a “cut and paste” mechanism in which the transposon is excised by the transposase upon expression and then relocates to a new locus by creating double strand breaks *in situ*. Most transposon systems used for gene delivery use a modified “cut and paste” system consisting of a transposon carrying the transgene of interest and a helper plasmid expressing the transposase (Figure 2). The “cut and paste” transposition mechanism involves recognition of the inverted terminal repeat sequences (IRs) by the transposase and excision of the transposon from the donor loci, usually a supplied plasmid. The two most commonly used transposon system for genetic modification of mammalian and human cells are *sleeping beauty* and *piggyBac*.

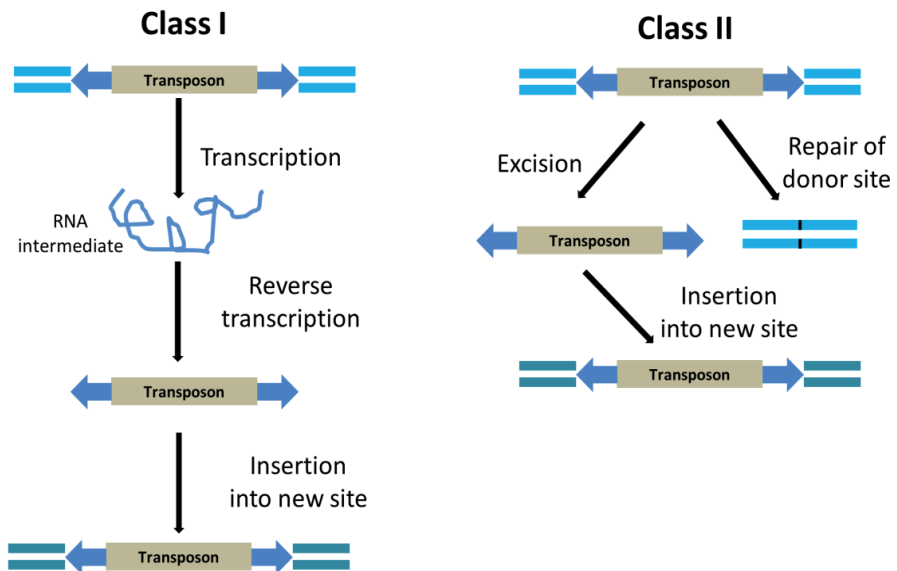


Figure 1. Class I and II transposons and mechanisms of integration.

The *sleeping beauty* (SB) transposon was reconstructed from the genome of salmonid fish using molecular phylogenetic data [3] and belongs to the Tc1/mariner superfamily of transposons. The sleeping beauty transposon is flanked by 230bp IRs which contain within them non identical direct repeats (DRs).

The *piggyBac* transposon was isolated from cabbage looper moth *Trichoplusia ni* [4]. One desirable feature of the *piggyBac* system is the precise excision of the transposon from the do-

nor site without leaving behind any footprints [5], making it an attractive feature for cellular reprogramming. Excision of the transposon from the donor site, creates complimentary TTAA overhangs which undergo simple ligation to regenerate the donor site bypassing DNA synthesis during transposition [6].

In “cis” delivery the transposase is carried by the same plasmid backbone as the transposon. In “trans” delivery it is delivered by a separate circular plasmid. For gene therapy purposes transposase and transposon are delivered either in “cis” or in “trans” (Figure 2). In “cis” delivery the transposase is carried on the same vector backbone as the transposon carrying the gene of interest (GOI). In the “trans” configuration, the transposase is delivered by a separate non integrating plasmid. The “cis” configuration has been shown to improve transposition efficiency [7], but there is a question of whether the linearized backbone carrying the transposase may also get integrated and lead to residual transposase expression. A comparison of the properties of *sleeping beauty* and *piggyBac* is described in Table 1.

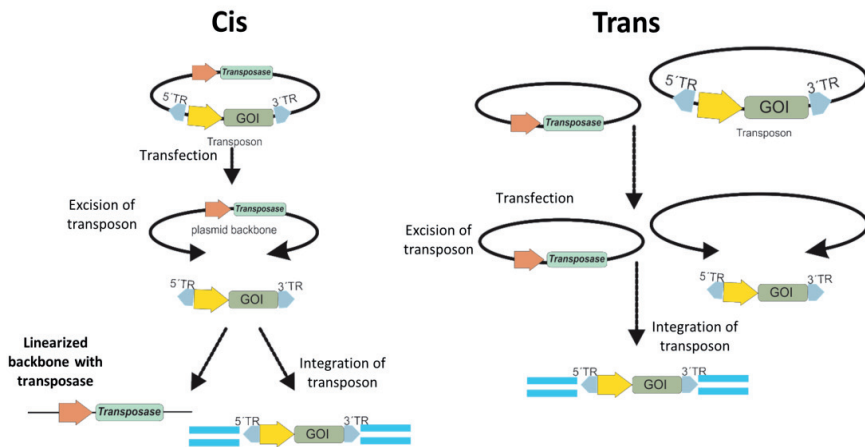


Figure 2. “Cis” and “Trans” transposon mediated gene delivery. GOI, gene of interest; 5’TR, 5’ terminal repeat; 3’TR, 3’ terminal repeat; the yellow and beige arrows indicate promoters to drive gene expression.

3. Advantages of transposon as gene delivery system

3.1. Lower cost compared to viral vectors

In spite of viral vectors having been successfully used in gene therapy clinical trials (e.g. generation of clinical grade T cells for immunotherapy [8], their use in extensive gene therapy regimens is constrained. Clinical grade viral vectors are very expensive to manufacture given the stringent regulatory oversight and limited number of GMP certified production fa-

cilities. A batch of clinical grade retroviral supernatant for treating patients costs between \$400,000 to \$500,000 (personal communciation, GMP facility director, Baylor College of Medicine). The production of clinical GMP (cGMP) grade viral supernatant is extremely time intensive as, in addition to optimization of culture conditions, the supernatant needs extensive testing for microbial contamination, presence of replication competent viral particles as well as validation of sequence and functionality. The entire production run and associated testing may require up to six months. These viral stocks also have limited shelf life. Upon release the desired cell type is transduced, selected and expanded which is then followed by quality assurance checks. This also requires extensive training of the personnel involved in production and testing and scaling up production as would be required for future gene therapy regimens will not be economical. In contrast, cGMP grade transposon plasmids can be manufactured more quickly. The production can be scaled up quickly and existing facilities can be upgraded and certified in a shorter time frame. The cost of manufacturing and release of cGMP grade plasmid DNA is between \$20,000 and \$ 40,000 [9]. The use of transposons drastically reduces both the time and cost of production of the gene delivery system. In the first clinical trial approved by the FDA for infusion of autologous *ex vivo sleeping beauty* modified T cells [10], the most time intensive step was the test for fungal and bacterial contamination (14 days).

	<i>sleeping beauty</i>	<i>piggyBac</i>
Cargo Capacity	~10 kb	>100 kb
Foot Print	Insertion site mutated upon excision	No "foot print" mutation
Needs titration for optimal activity	Yes	Yes
Hyper Active Versions	SB100X (most active SB version)	hyPBace
Effect of 'N' and 'C' terminal modifications	50% or more reduction in efficacy	No apparent reduction in efficiency
Integration site preference	More random	Slight increased preference for genes and TSS
Can be engineerd to bias integration sites	Yes	Yes

Table 1. Comparison of *sleeping beauty* and *piggyBac* properties. TSS, transcriptional start sites.

3.2. Delivery of large and multiple transgenes

Although retroviral and lentiviral vectors have been successfully used for delivering multiple transgenes, they are limited by their cargo capacity[11,12]. Both these vector systems can carry a limited cargo of up to 8kb which is limited by the packaging capacity of their capsid envelop [13]. Early reports demonstrated the *sleeping beauty* system to have reduced efficiency beyond transposon size of 10kb [14]. In contrast the *piggyBac* system has been successful-

ly utilized to modify primary human lymphocytes with 15 kb transposon with an initial transfection efficiency of 20% which increased up to 90% upon selection and expansion [15]. The *piggyBac* system has been successfully used for mobilizing transposons as large as 100 kb in mouse embryonic stem (ES) cells [16]. An increased cargo capacity also imparts the ability to deliver multiple transgenes to the same cell. For example, using the *piggyBac* system, human cells were efficiently modified to express a three subunit functional sodium channel which retained its electro-physiological properties even after 35 passages [17].

3.3. Less immunogenicity

One of the major concerns for viral gene delivery system is the associated immunogenicity as evidenced by the death of a patient receiving liver targeted adenoviral gene therapy for partial ornithine transcarbamylase deficiency in 1999 [13]. The systemic delivery of the viral particles initiated a cytokine storm leading to multiple organ failure within four days of administration of the vector [18]. Attempts have been made to reduce the immunogenicity of viral vectors by stripping them of all endogenous viral genes ('guttled' or 'helper-dependent' vectors) [19], but even the use of modified viral delivery systems are potentially immunogenic as evidenced by long term inflammation of rat brains injected with replication deficient adenoviral vectors [20].

Transposons are circular plasmid DNA molecules and do not contain a viral shell or viral antigens. The host response to non-viral vectors has not been well characterized. Toll-like receptor (TLR)-9 is known to recognize DNA with unmethylated CpG dinucleotides in the endosome which can lead to signalling via MyD88 and production of inflammatory mediators such as TNF and IFN- α [21]. Other mechanisms of innate immune sensing of naked DNA include DNA-dependent activator of interferon (IFN)-regulatory factors (DAI) (also called Z-DNA-binding protein 1, ZBP1), RNA polymerase III (Pol III), absent in melanoma 2 (AIM2), leucine-rich repeat (in Flightless I) interacting protein-1 (Lrrfip1), DExD/H box helicases (DHX9 and DHX36), and most recently, the IFN-inducible protein IFI16 [22]. These molecules use independent and sometimes overlapping signalling pathways to elicit immune response to delivered DNA. Nonetheless, much remains to be discovered about host immune response to delivered DNA and how to overcome such an obstacle for effective gene therapy.

3.4. Less propensity for oncogenic mutations

Human immunodeficiency virus (HIV) has been shown to prefer genes for integration in SupT1 and Jurkat cells [23]. Murine leukemia virus (MLV) derived vectors have been used for stable gene transfer for therapy but they have been shown to prefer transcriptional start sites (TSS) for integration [24]. Integrations near the promoter of the LMO2 proto-oncogene has been associated with leukemia in the French X-SCID gene therapy trial [25]. The genome wide mapping of *sleeping beauty* transposons in mammals have revealed a modest bias towards transcriptional units and upstream regulatory sequences which varies between cell types [26]. The integration site profiling of both *piggyBac* in primary human cells and cell lines have revealed no preferred chromosomal hotspots [7,27]. It also has no preference for genomic repeat elements and known proto-oncogenes. *PiggyBac* has a preference for inte-

grating into RefSeq genes and near TSS and CpG enriched motifs although this may be influenced by the state of the cell or type of the cell. Both *sleeping beauty* and *piggyBac* are being engineered for site-directed gene delivery to improve the safety of gene transfer. True genotoxic risk for viral vectors was not discovered until they were used in humans. Transposons have not yet been used in humans, though one clinical trial has been approved.

4. Challenges of transposon as gene delivery system

Given the promise of transposons as gene delivery vehicle, it suffers from certain challenges e.g. reduced delivery, random integration profile and silencing of the integrated transgene.

4.1. Low delivery efficiency

Transposon systems are carried by naked DNA plasmids and their efficiency is limited to the efficiency of getting the plasmid into the cell by chemical or physical means. Certain primary cells and cell lines are easy to transfect (e.g. HEK293, HeLa, Hepatocytes) and transposons have high transposition efficiency in these cells. But other clinically relevant cells (e.g. primary lymphocytes) are difficult to transfect. Often the method used for transfection (e.g. nucleofection and electroporation) is toxic to the cells and leads to excessive cell death thus reducing the efficiency of stable transfection. Efforts are on to circumvent these difficulties by developing novel delivery methods e.g. cell-penetrating peptides (CPP)–*piggyBac* fusions [28] or using polyethylenimine [29]. Some investigators have encapsulated transposon systems within viruses to use the virus to deliver the DNA from which transposition occurs [30–34] This may improve efficiency, however, the issues with immunogenicity of viruses remain.

4.2. Random integration profile

Transposons as described above have uncontrolled or relatively random integration preference with regards to genomic elements. This leaves the transposed transgene open to influence of the neighboring genomic region. Additional, uncontrolled or not site-directed integration increases the risk for possible genotoxicity.

4.3. Silencing of the integrated transgene

Gene silencing has been observed when using *sleeping beauty* in cultured cells [35]. Transgene silencing and epigenetic transgene modification has not been well studied with *piggyBac*.

5. Applications

Both *sleeping beauty* and *piggyBac* have demonstrated correct of disease phenotypes in animal models or in human cells (Table 2).

Disease	Transposon system	Reference
Hemophilia B	SB	[34,36]
Hemophilia A	SB	[37,38]
Tyrosinemia Type I	SB	[39]
JunctionalEpidermolysisBullosa	SB	[40]
Diabetes	SB	[41]
Huntington's disease	SB	[42]
Mucopolysaccharidosis I & VII	SB	[43,44]
α 1-antitrypsin deficiency	PB	[45]

Table 2. List of diseases corrected with *Sleeping Beauty* (SB) and *piggyBac* (PB)

5.1. Genetic modification of human T lymphocytes

Peripheral blood and umbilical cord T cells have been extensively modified with both viral and non-viral gene delivery systems for immunotherapeutic purposes [10]. This therapeutic avenue has been successfully used for the treatment of viral infections and Epstein Barr virus (EBV) associated lymphoma post autologous bone marrow transplantation [46,47]. They also hold promise for treatment of other cancers [48-50]. But the use of viral vectors for the generation of clinical grade T cells is expensive, time intensive and not free of risks. Non-viral gene delivery systems, including DNA transposons, are being increasingly explored as an alternative strategy.

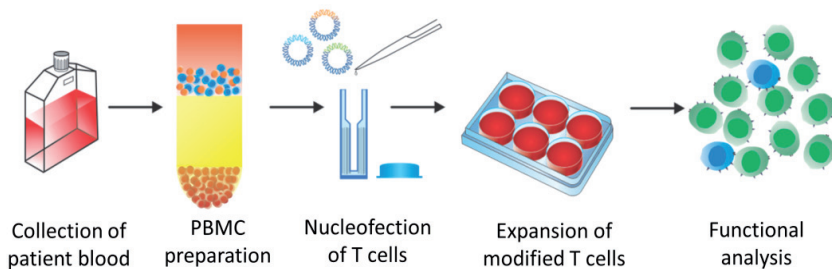


Figure 3. Schematic of transposon modification of primary human T cells.

A schematic of how primary human T lymphocytes can be gene modified with transposons is shown in Figure 3. The *sleeping beauty* system was used to successfully modify peripheral blood mononuclear cells with a CD19-specific chimeric antigen receptor (CAR)[9]. These modified PBMCs were then used to generate CAR⁺ T cells which preserved their CD4⁺, CD8⁺, central memory and effector-effector cell phenotypes. The *piggyBac* system has also

been optimized to achieve stable transgene expression in human T lymphocytes [51]. Further, primary lymphocytes have been modified with multiple transgenes to redirect their specificity for CD19 and make them resistant to off target effects of chemotherapeutic drugs like rapamycin [15]. Cytotoxic T lymphocytes specific for Epstein Barr Virus (EBV) have also been successfully modified with human epidermal growth factor receptor-2 specific CAR (HER2-CAR)[52]. The first clinical trial involving transposon modified autologous T cells with a second generation CD19-specific CAR has been approved by the Food and Drug Administration[10]. This trial will involve the infusion of *ex vivo* expanded autologous T cells in patients undergoing autologous hematopoietic stem cell (HSC) transplantation with high risk of relapsed B-cell malignancies.

5.2. Generation of induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) generated from a patient's own differentiated somatic cells holds promise for regenerative medicine. Early successful attempts involved delivery of defined reprogramming factors using retroviral vectors [11,53]. Unfortunately 20% of the chimeric offspring obtained from germline transmission of retrovirally reprogrammed clones developed tumors due to reactivation of the c-myc oncogene [54]. In addition, ectopic expression of the reprogramming factor(s) has been linked to tumors and skin dysplasia [55-56]. One way to circumvent the use of viral delivery systems is to deliver the programming factors as recombinant proteins [57] or by repeated plasmid transfections [58], both of which have proven to be extremely slow and inefficient. The higher gene delivery efficiency of transposons together with their ability of being excised from the cells post reprogramming and differentiation make them an attractive choice for generating iPSCs.

Somatic cells have been transfected with *piggyBac* transposons carrying reprogramming factors and transposase. Reprogrammed iPSCs are selected and propagated to obtain individual iPSC clones. To generate transgene-free iPSCs, the transposase is re-expressed to remove the reprogramming factors followed by negative selection to identify transgene-free iPSCs (Figure 4).

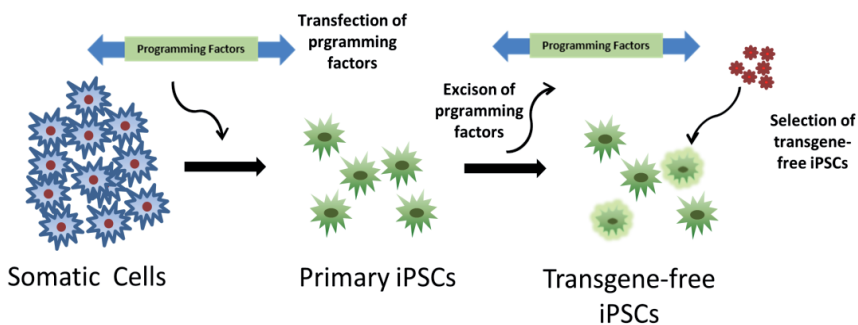


Figure 4. Generation of transgene-free iPSCs using the *piggyBac* system.

The *piggyBac* system seems to be ideally suited for this as it can undergo precise excision and does not leave behind “foot print” mutations [5]. In contrast, the *sleeping beauty* system has been shown to excise imprecisely leaving behind altered insertion sites [3]. The *piggyBac* system has been successfully used to generate transgene free iPSCs from both mouse and human embryonic fibroblasts with efficiency comparable to retroviral vectors [59-60]. *PiggyBac* has also been used to successfully reprogram murine tail tip fibroblasts into fully differentiated melanocytes which are more compatible with cell therapy regimens [61]. The use of a *piggyBac* based inducible reprogramming system also proved to be more stable and quicker than an inducible lentiviral system [62].

5.3. Genetic modification of stem cells

Transposons have been used for genetic modification of human embryonic stem cells [63]. More recently, transposons have been used to insert bacterial artificial chromosomes (BACs) in human ES cells [64]. Both *sleeping beauty* and *piggyBac* have been used to genetically modify hematopoietic stem cells [65]. Transposons provide an effective mechanism for permanent (or reversible in the case of *piggyBac*) genetic modification of a variety of stem cell types for eventual use in therapy.

6. Current hot topics and future directions

6.1. Generation of hyperactive transposon elements

SB100X and native *piggyBac* both have similar activity levels in human cells which is 100 fold more than the native *sleeping beauty*. The hyperactive *piggyBac* transposase (hyPB) has been shown to have 2 to 3 fold more activity than SB100X or native PB [66] (Figure 5).

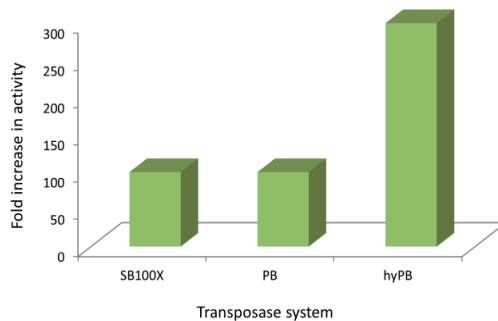


Figure 5. Comparison of transposase activity in human cells

Efficiency of transposition is perceived as a bottleneck to efficient gene delivery. Attempts to engineer hyperactive versions of transposase have resulted in versions with increasing transposition activity. Strategies employed include import of amino acids from related transposases [67], alanine scanning [68] and site directed mutagenesis [69]. The construction of the SB100X transposase with ~100 folds higher activity than the original *sleeping beauty* transposase employed a high throughput screen of mutant transposases obtained from DNA shuffling [70]. A hyperactive version of the *piggyBac* transposase (hyPBase) has also been engineered with 17-fold increase in excision and 9-fold increase in integration [71]. The hyPBase has 7 amino acid substitution identified from a screen of PBase mutants but none of the 7 substitutions are in the catalytic domain of the transposase. The hyPBase also has footprint mutation frequency (<5%) comparable to the wild type transposase and no apparent effect on genomic integrity. Unlike SB100X which showed a 50% reduction, the addition of a 24 kDa ZFN tag did not significantly alter transposition efficiency [66]. In vivo, a mouse codon optimized version of hyPBase showed 10-fold greater long term gene expression than both native *piggyBac* and SB100X.

6.2. Engineering transposon systems for site-directed integration

Random integration of transgene during delivery have resulted in adverse events including leukemia [25,72]. Integration of transgenes at other genetic loci may also affect expression of critical genes. Engineering transposon systems for site-directed integration would allow transgene delivery to sites in the genome resulting in improved gene expression, reduced positional effects at the site of integration, and improved safety. Most studies have utilized fusion of DNA-binding domains to the transposase to achieve site directed integration, beginning with the engineering of the *sleeping beauty* system. *Sleeping beauty* has been engineered to bias integration into plasmids containing target sites [73-74] and near selected elements and repeat elements in the genome [75-76]. The *piggyBac* system seems to be more suited for transposase modifications as the addition of additional domains to the transposase does not alter the systems efficiency [7,77-79]. A Gal4-*piggyBac* fusion transposase has been shown to bias integration near Gal4 sites in episomal plasmids [80] and the genome [81]. A chimeric transposase containing an engineered zinc finger protein (ZFP) fused to the native *piggyBac* transposase has also been successfully used to bias integration at the genomic level [79]. Researchers have also used transcription factor DNA binding domains fused to the *piggyBac* transposase to label nearby transcription factor binding sites in the genomes of cells [82]. Current approaches are hampered by the ability of the transposase to integrate on its own without the targeting machinery which can lead to off-target integration. Further engineering modifications to both the transposase and transposon may overcome this limitation.

7. Conclusion

Transposon systems are well suited for *ex vivo* gene therapy and *in vivo* delivery to target organs may also become a reality in the future. The advantages of lower cost and more

widespread applicability than viral vectors, in combination with the potential for site-directed gene delivery, make transposons a promising non-viral gene delivery system as an alternative to viral vectors.

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Lentiviral Gene Therapy Vectors: Challenges and Future Directions

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Additional information is available at the end of the chapter

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1. Introduction

Lentiviral vectors (LV) are efficient vehicles for gene transfer in mammalian cells due to their capacity to stably express a gene of interest in non-dividing and dividing cells. Their use has exponentially grown in the last years both in research and in gene therapy protocols, reaching 12% of the viral vector based clinical trials in 2011 [1]. This chapter reviews and discusses the state of the art on the production of HIV-1- based lentiviral vectors.

1.1. Lentiviruses

Lentiviruses are human and animal pathogens that are known to have long incubation periods and persistent infection. The time between the initial infection and the appearance of the first symptoms can reach several months or years [2]. Nowadays lentiviruses are classified as one of the seven genus of *Retroviridae* family. *Lentivirus* genus is composed by nine virus species that include primate and non-primate retroviruses (Figure 1) [3].

All Retroviruses share similarities in structure, genomic organization and replicative properties. Retroviruses are spherical viruses of around 80-120 nm in diameter [4] and are characterized by a genome comprising two positive-sense single stranded RNA. Also, they have a unique replicative strategy where the viral RNA is reverse transcribed into double stranded DNA that is integrated in the cellular genome [5]. Together with the RNA strands, the enzymes necessary for replication and the structural proteins form the nucleocapsid. The later is inside a proteic capsid that is surrounded by a double lipidic membrane [6]. Connecting the lipidic membrane and the capsid there are the matrix proteins. The lipidic membrane has its origin in the host cells and presents at surface the envelope viral glycoproteins (Env) (Figure 2).

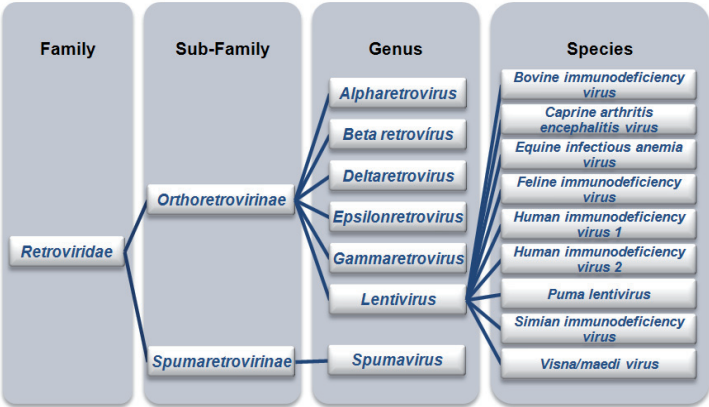


Figure 1. Lentiviruses taxonomy by the International Committee on Taxonomy of Viruses (ICTV).

Within the *Retroviridae* family, retroviruses can be classified as simple or complex. The complex retroviruses include the lentiviruses and spumaviruses presenting a more complex genome with additional regulation steps in their life cycle.

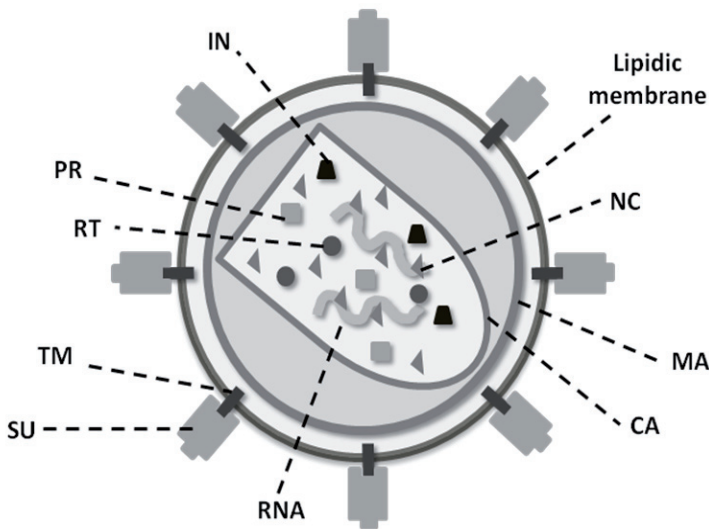


Figure 2. Schematic representation of a retrovirus particle. Abbreviations: NC – nucleocapsid; MA – matrix; CA – capsid; SU – surface subunit of Env protein; TM – transmembrane subunit of Env protein; RT – reverse transcriptase; PR – protease; IN – integrase.

1.2. HIV-1 genome

HIV-1 genome has about 9-10 kb and is constituted by several non-coding sequences that control gene expression and protein synthesis, and genes that code for regulatory and accessory proteins in addition to the structural and enzymatic genes *gag*, *pol* and *env*, common to all retroviruses (Figure 3).

The *gag* gene codes for a polypeptide that is proteolytically cleaved by the viral protease (PR) originating three main structural proteins: matrix (MA), capsid (CA) and nucleocapsid (NC). The *pro* gene codes for a polypeptide that after cleavage by PR, during the virus maturation, originates PR, reverse transcriptase (RT) and integrase (IN). These enzymes play critical roles in the life cycle of retroviruses since their functions are the cleavage of viral polypeptides (also involved in virus maturation), the reverse transcription of viral RNA to double-stranded DNA (provirus) and the integration of the provirus into the cellular genome [7]. Finally the *env* gene encodes a polypeptide that is cleaved by cellular proteases in two proteins, the SU (surface) and TM (transmembrane) subunits. Together, these two proteins are the structural units of the Env protein that will interact with cellular receptors of the host cell allowing for virus entrance into the cell [10].

Flanking the retroviral provirus there are the 5' and 3' Long Terminal Repeats (LTRs) composed by the 3' untranslated region (U3), repeat elements (R) and 5' untranslated region (U5). The LTRs contain the enhancer/promoter sequence that allows for gene expression, the att repeats important for provirus integration and the polyadenylation signal (polyA).

The HIV-1 genome also has other six genes that code for two regulatory proteins (Tat and Rev), and four accessory proteins: Vif, Nef, Vpr, and Vpu. Tat protein interacts with cellular proteins and the mRNA TAR sequence acting by increasing the viral transcription hundreds of times. Rev interacts with Rev Responsive Element (RRE), a cis-acting sequence located in the middle of the *env* gene allowing the efficient nuclear export of unspliced or singly spliced messenger RNA. The functions of accessory proteins are related with pathogenesis of the virus and they are not crucial for the viral replication *in-vitro*.

The function of all HIV-1 proteins and their interactions with the host cells are not yet clearly understood but it is already reported that there are 2589 unique HIV-1-to-human protein interactions that are formed by 1448 human proteins [8,9].

Additionally to the coding sequences, the lentivirus genome also has several non-coding cis-acting sequences that play important roles in viral replication. The LTRs contain the Transactivator Response element (TAR) for the interactions of the complex formed by the Tat protein and transcriptional factors. After the 5' LTR there are the primer binding site (PBS), where the reverse transcription starts, and the packaging signal (Ψ). Within the *pol* sequence there are also the central polypurine tract (cPPT) and the central termination sequence (CTS) contributing both for the efficient reverse transcription. Further there are the RRE in the middle of *env* gene and near the beginning of the 3' LTR the polypurine tract (PPT), a purine rich region where the synthesis of the plus strand DNA during the reverse transcription starts [10].

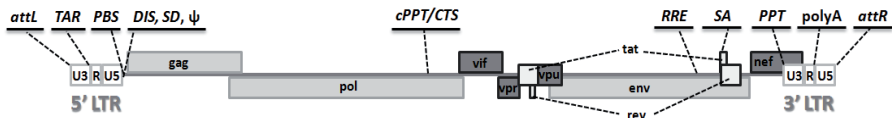


Figure 3. Schematic representation of HIV-1 provirus genome. Abbreviations: LTR - long terminal repeat; attL and attR - left and right attachment sites; U3 - 3' untranslated region; R - repeat element; U5 - 5' untranslated; TAR - transactivation response element; PBS - primer binding site; DIS - dimerization signal; SD - splice donor site; SA - splice acceptor site; ψ - packaging signal; cPPT - central polypurine tract; CTS - central termination sequence; RRE - Rev response element; PPT - 3' polypurine tract; polyA - polyadenylation signal.

1.3. HIV-1 life cycle

The HIV-1 Life cycle starts when the Env glycoproteins GP120 located at surface of the viral envelope bind the CD4 cellular receptor and co-receptor CCR5, CXCR4 or both. This binding induces conformational changes of Env glycoproteins that allows for the fusion of the viral envelope with the cell membrane and the consequent entry of the viral core into the cell. Once inside the cell the capsid starts to disintegrate and the RT enzyme begins the reverse transcription where a double-stranded proviral DNA is synthesized using one of the positive single-strand viral RNA molecules as template. When reverse transcription is completed the double-strand DNA now called provirus forms a complex with viral proteins (RT, IN, NC, Vpr and MA) and cellular proteins termed pre-integration complex (PIC) that is imported to the cell nucleus by an ATP-dependent manner. It is this energy-dependent mechanism that allows the transduction of non-dividing cells by lentiviruses, unlike γ -retrovirus.

In the nucleus the linear provirus is integrated into the cellular genome by the integrase. Now all the requisites to produce new viruses are filled and the proviral DNA is transcribed into mRNA by the cellular RNA polymerase II. Still inside the nucleus some transcripts suffer a splicing event. The mRNA transcripts are exported from the nucleus to cytoplasm to be transcribed and to start to form the viral particles; two full-length RNA transcripts will be packaged in the viral particles.

The assembly of the viral proteins and the viral RNA occurs near the cellular membrane, in specific regions called lipid-rafts that are rich in cholesterol and sphingolipids. The immature viral particles are released from cells by budding. After leaving the cells, the viral protease cleaves the Gag and Pol proteins precursors to finally generate a mature infectious virion (reviewed by [5,10]).

2. Lentiviral vector development

The development of lentiviral vectors (LVs) started in 1989 when an HIV-1 provirus with *achloramphenicol acetyltransferase (cat)* reporter gene in place of the non-essential *nef* gene was constructed. The transfection of Jurkat cells with this modified provirus plasmid produced infectious replicative competent viruses, very similar with wild-type HIV-1, that could be used

as a tool for study HIV infection [11]. Few months after, the same group presented the first replication-defective HIV-1 vector. In a trans-complementation assay for measuring the replicative potential of HIV-1 envelope glycoprotein mutants they used an identical HIV-1 provirus construction but with a deletion in the *env* gene. The Env glycoproteins were supplied by an independent expression plasmid. The co-transfection of these two plasmids allowed for the production of replication-defective viruses [12]. These vectors were structural identical to the wild-type virus, but lacked in their genome the *env* gene. They could only perform a single cycle of replication because their host cells, after infection, did not have the *env* gene to produce infectious virus. Although the principal aim of these studies was not the creation of viral vectors, they were the basis of lentiviral vector development, suggesting that lentiviruses could be adapted as a tool for genetic material transfer and permanent modification of animal cells.

Other preliminary studies were being conducted and several important discoveries or innovations had also contributed for the development of LVs. The introduction of the resistance marker gene *hypoxanthine-guanine phosphoribosyl-transferase (gpt)* under the expression control of SV40 promoter in the place of *env* gene deletion allowed the first quantification of infectious LVs produced [13]. Like it had been observed for other γ -retroviral vectors (γ -RVs) it was possible to produce infectious lentiviral particles with Env glycoproteins from other viruses (pseudotyping); for example the Moloney Murine Leukemia Virus amphotrophic envelope 4070A (A-MoMLV) [13], and Human T-cell Leukemia Virus Type I (HTLV-I) envelope [14] were successfully used suggesting that *env* gene was not necessary for virion particle formation. The localization and sequence of packaging signal was identified as the main responsible for the packaging of viral RNA [15] suggesting that modified RNAs with Ψ could also be packaged into virions. The discovery of the great stability conferred to LV pseudotyped with Vesicular Stomatitis Virus-G protein protein (VSV-G) allowed to concentrate the LV up to 10^9 by ultracentrifugation or ultrafiltration without significant loss of infectivity [16,17]. It was shown that LVs can transduce efficiently non-dividing cells, their principal advantage over the oncoretroviral vectors [16,18,19].

All these steps showed the potential of using modified lentiviruses as vectors, stimulating the iterative studies and the evolution of LVs in the next years. Their further development was based in safety principles (most of them already used in the development of oncoretroviral vectors) such as the splitting of the genome into several independent expression cassettes: the packaging cassette with the structural and enzymatic elements, the transfer cassettes with the gene of interest and the envelope expression cassette. In addition, the elimination of non-essential viral elements and the homology reduction among the expression cassettes also contributed to avoid the possibility of recombination, vector mobilization and the generation of replicative competent lentiviral vectors (RCLVs).

2.1. Four generations of packaging constructs

Four generations of LVs are currently considered; these differ from each other in terms of the number of genetic constructs used to drive the expression of the viral components, the number of wild-type genes retained as well as the number and type of heterologous *cis*-elements used to increase vector titers and promote vector safety.

The system of three expression cassettes developed in 1996 by Naldini *et al.* [16] is considered the first generation of LVs. In this system the packaging cassette has all structural proteins, with exception of Env glycoproteins, and all accessory and regulatory proteins. Later the 5' LTR was substituted by a strong promoter (CMV or RSV) and the 3' LTR by an SV40 or insulin poly(A) site to reduce the homology between the cassettes. To prevent the packaging of viral elements the Ψ and PBS were deleted. In the *env* expression cassette the gp120 from HIV-1 was replaced by other *env* genes as VSV-G or amphotrophic MLV envelope (Figure 4). Finally the transgene cassette was composed by the 5' LTR, the ψ with a truncated *gag* gene, the RRE cis-acting region and the gene of interest under the control of a heterologous promoter (usually CMV) and the 3'LTR [16,20]. This system allowed in an easy way to achieve good titers but its level of safety was not very high. RCL could be generated just with three recombination events by homologous recombination between the viral sequences in all cassettes or endogenous retroviral sequences in cells. In order to improve the safety and decrease the cytotoxicity of LVs, the three plasmid system was maintained, but all accessory genes not required for viral replication *in vitro* (*vif*, *vpr*, *vpu*, and *nef*) [21] were removed without negative effects on vector yield or infectivity. And in this way the second generation of LVs was developed (Figure 4) [22–25]. In the second generation, if by chance some RCL was generated, it would be unlikely to be pathogenic [26]. However the number of homologous events to generate RCL was the same as in the previous generation.

Reducing the lentiviral sequences by eliminating the *tat* and place the *rev* in an independent plasmid was the further step that originated the third generation of LV [27]. The *tar* sequence was replaced by a strong heterologous promoter. Therefore Tat protein was no longer necessary to increase the transgene transcription and *tat* gene was eliminated. This contributed for the reduction of lentiviral elements in the constructs. Rev was placed in an independent non-overlapping plasmid increasing the safety since now four events of homologous recombination were required for RCL formation [27]. With these new features, the vectors of third generation (Figure 4) presented a higher level of biosafety and, as the titers did not decreased, their use was widespread. Today they are the most commonly used LVs.

Although the formation of RCL was improbable, homologous recombination between the constructs was still possible since RRE sequence and part of packaging sequence in *gag* gene was in both transfer and structural packaging constructs. To solve these problems other solutions were developed originating the fourth generation of LV. The first approach used consisted in the replacement of the RRE sequences by heterologous sequences with similar functions that do not need the Rev protein. Some of these sequences were the Mason-Pfizer monkey virus constitutive transport element (CTE), the posttranscriptional control element (PCE) of the spleen necrosis virus and the human nuclear protein Sam68 [28–31]. The heterologous sequences increased the stability of the transcripts allowing their nuclear export. However the titers have decreased.

In 2000 a different approach based on codon optimization was implemented in lentiviral vector design [33]. This approach consists in perform silent mutations, changing the codon that codes for a certain aminoacid for other that codes for the same aminoacid, in principle, with a higher intracellular availability [32]. Applying this to the packaging and transfer con-

structs the homology between them was eliminated. These changes also allowed an independent expression of Rev since the sequences with suboptimal codon usage in HIV-1 genome, that conferred RNA instability and consequently lower expression, disappeared [32]. In the fourth generation (Figure 4) the homology between constructs were severely reduced but the titers had also been affected comparing with systems with the Rev/RRE [32]. Also, with the independence of Rev/RRE system, the level of biosafety decreased since the number of homologous recombination events for RCL formation was again three. Maybe due to these drawbacks the fourth generation has not been extensively used. However the codon optimization technology had been used to decrease the homology between sequences, improve the expression of viral components and viral titers [33].

Regarding the biosafety concerns about RNA mobilization and the possibility of generating RCLs, other improvements in packaging constructs were used and tested in transient LVs productions. These improvements relied on the concept of *split-genome* used for retroviral and lentiviral vector development but this time applied to the packaging construct. The *gag-pol* sequences were divided by two or three independent expression cassettes, disarming the functional *gag-pol* structure that is essential for vector mobilization [34]. In these systems additional recombination events between the several expression cassettes are necessary to generate RCLs which seems to contribute to a significant decrease of recombinant vectors formation with a functional *gag-pol* structure [35,36]. Although this increased LV safety the transduction efficiency and the LV production are challenged by the higher number of plasmids required [37].

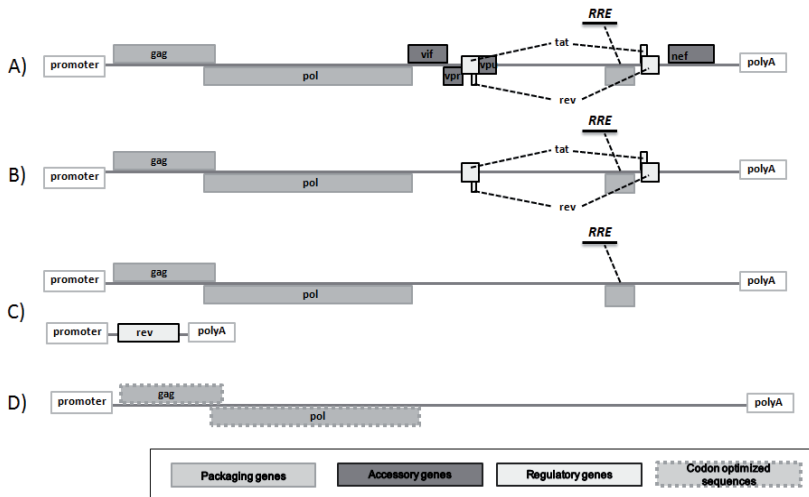


Figure 4. Schematic representation of the four generations of lentiviral packaging constructs: A) First generation packaging vector. B) Second generation packaging vector. C) Third generation packaging vector. D) Fourth generation packaging vector.

2.2. Transfer vector

The transfer vector is the expression cassette of the transgene that will be packaged into the viral vector and integrated in the cellular genome of the target cells. Besides the gene of interest and the commonly heterologous promoter for transgene expression, the transfer expression cassette must have: the sequences responsible for the expression of the full-length transcript and its packaging into the newly formed virions in the producer cells; the sequences that will interact with viral and cellular proteins to allow an efficient reverse transcription, transport into the cellular nucleus and proviral integration into target cells genome. Despite the simple design and the lack of sequences that code for viral proteins, the transfer vector also evolved over the time. This evolution was primarily focused on safety by reducing and replacing the viral sequences by heterologous elements and in optimizing both safety and efficiency by the addition of several *cis*-acting elements to the transfer cassette [10].

The transfer vectors usually used in the first and second generation of packaging constructs LVs were composed by the 5' LTR which include the TAR sequence, the PBS, the SD, the Ψ , the 5' part of *gag* gene, the RRE sequence, the SA, an heterologous promoter followed by the gene of interest, the PPT and polyA within the 3' LTR. The first hundreds of base pairs of *gag* are included after the packaging signal to increase the packaging efficiency (Figure 5). To avoid *gag* translation the initiation codon is usually mutated or cloned out of frame [16,20]. However, like it was previously found for γ -RVs, this transfer vector design with both wild-type LTRs can lead to integration genotoxicity and facilitates the mobilization of the transgene in the case of posterior infection of transduced cells [38]. To overcome these biosafety problems the LTRs of the transfer vector suffered additional changes. One of the first modifications was the replacement of the enhancer/promoter and Tar sequence of the 5' LTR by a strong heterologous promoter allowing the transcription of the full-length viral RNA in a Tat-independent manner [25]. In addition the wild-type enhancer/promoter sequences in the U3 region of the 3' LTR were deleted originating the self-inactivating (SIN) LVs [27,39,40], as it had already been done for γ -RVs [41].

The SIN design (Figure 5) generates in the target cells a proviral vector without enhancer/promoter sequences in both LTRs. In producer cells the packaged RNA transcript does have the heterologous promoter in the 5' end. Afterwards, in the target cells, during the reverse transcription, the U3 region of 3' LTR is copied and transferred to the 5' LTR. This transcriptional inactivation offered by the SIN design presents several safety advantages: prevents the formation of potentially packageable viral transcripts from the 5' LTR and consequently prevents vector mobilization by prior infection with a replicative retrovirus [39,42]; reduces the risk of insertional mutagenesis induced by the transcriptional interference of the LTRs in the neighboring sequences that can lead to the activation or up-regulation of oncogenes [43]; and lowers the risk of RCL formation by the reduction of the sequences with homology with wild-type virus.

The adoption of SIN design did not affected LV production as it happened with γ -RVs [27,39,40,44]. However both LVs and γ -RVs displayed high frequencies of read-through of the 3' polyadenylation signal which can lead to the transcription of cellular sequences as oncogenes. This inefficient termination of transcription could suggest that some of the enhanc-

er/promoter sequences deleted can have a role in an efficient transcription termination [45]. In this context several improvements were done by the addition of heterologous elements to increase safety, expression and efficacy of LVs: heterologous polyadenylation signals in the 3'LTR could increase the efficiency of LVs and are particularly beneficial in the case of SIN LVs avoiding the read-through of cellular genes [40,46]; the chromatin insulators as the chicken hypersensitive site 4 (cHS4) sequence core from the β -globin locus control region (LCR) can reduce the interference from the neighboring regions in the vector and transgene expression [48]. Also these can improve the LV safety avoiding the full-length vector transcription or reducing long-distance effects of the integrated transgene promoter on neighboring cellular genes in the target cells. Additionally to the increased safety, insulators can help to maintain the gene expression over time preventing transcriptional silencing events in both producer and target cells [47–49]; incorporation of certain post-transcriptional regulatory elements (PRE) like the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) near the 3' untranslated region can also decrease the read-through in SIN vectors increasing the transgene expression and viral titers, [50–53]. The firsts WPRE sequences used contained part of a sequence that codes for a protein (WHV X) that has been reported a few times as related with carcinoma formation, posing safety concerns. However a further improved WPRE was created without this potential harmful sequence [54]; The cPPT sequence contributes for efficient reverse transcription and the proviral nuclear import processes. Although this non-essential sequence was not used in the firsts transfer vectors, its re-insertion increased the gene transfer efficiency [55–57].

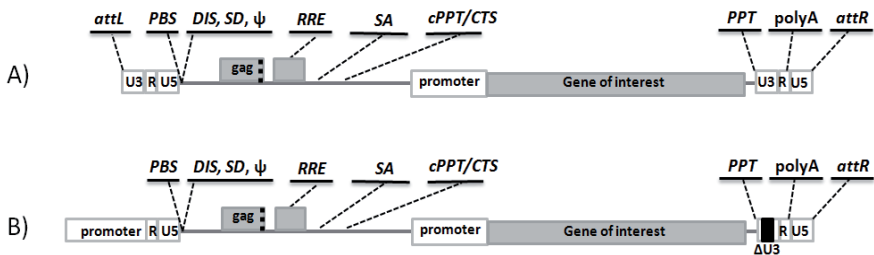


Figure 5. Schematic representation of a non-SIN transfer vector (A) and a SIN transfer vector (B).

3. Pseudotyping

LVs, as other retroviral vectors, can incorporate in their viral particles Env glycoproteins from other enveloped viruses, a feature denominated pseudotyping. This was firstly demonstrated for the HIV-1-based lentiviral vector using a Moloney Murine Leukemia Virus amphotropic envelope 4070A (A-MoMLV) [13] and an Human T-cell Leukemia Virus Type I (HTLV-I) envelope [14].

In general the pseudotyped LVs have the tropism of the virus where glycoproteins are derived from, but there are some exceptions such as the glycoprotein of the Mokola virus, where the pseudotyped vectors did not presented the specific neurotropism of the parental virus [58]. This ability of LVs to be pseudotyped showed to be advantageous since several glycoproteins could be tested to improve the transduction of cells with different receptors. As an example, HIV-based LVs pseudotyped with glycoprotein derived from the Rabies virus PV strain exhibited a great efficiency and neuronal tropism among the tested envelopes [59].

In addition to the tropism of LVs, the Env glycoproteins also affect vector structure and stability, the interactions with the target cells and the LV behavior during the infection. One example is LVs pseudotyped with rabies virus glycoprotein which allow for the retrograde axonal transport and access to the nervous system after peripheral infection [60]. Another example is the stability conferred to LVs by the VSV-G glycoproteins. The VSV-G glycoproteins are one of the most used Env proteins due to their wide tropism, with high titers achieved, great stability and resistance conferred to the LVs that allows for their concentration by ultracentrifugation. In addition they resist to freeze-thaw cycles, an important factor for storage of the vectors [16,18,19,61]. Despite these positive characteristics, the VSV-G protein is toxic for producer cells if expressed constitutively [17] and is inactivated by human serum complement [62], although this inactivation can be minimized using VSV-G conjugated with poly(ethylene glycol) [63].

Up to the present, several glycoproteins were used to pseudotype LVs (Table 1) each one presenting specific advantages and disadvantages that also depend on the LV application.

Although LVs pseudotyped with different Env glycoproteins present different tropisms, being some tropisms more selective than others, in general these are not specific for a particular cell type as happens with HIV-1 glycoproteins [80,81]. For instance, the Ebola Zaire (EboZ) glycoprotein seems to be superior to other glycoproteins in the transduction of apical airway epithelia [72]. However also has been shown to transduce liver, heart, and muscle tissues [82].

This lack of specificity is not ideal from a clinical point of view, especially for *in vivo* gene therapy applications since it can lead to the infection of cells that do not need to be transduced [83].

Several strategies have been used to increase the specificity of infection in order to retarget the LVs to a cell of interest. These strategies consisted in genetic engineering of virus envelopes by deletion of some domains or fusing molecule-ligands (growth factors, hormones, peptides or single-chain antibodies) in several locations of the viral glycoproteins. The purpose is to choose cellular receptors specifically expressed on the target cells that will interact with the chimeric glycoproteins, restricting this way the vector tropism. A successful example was the removal of the heparan sulfate binding domain from the Sindbis virus envelope protein which effectively restricted the tropism of pseudotyped LVs to dendritic cells. This genetic modified Env protein interacts solely with the C-type lectin-like receptor almost exclusively on primary dendritic cells unlike its natural counterpart [84].

Species/Envelope	Vectors	Comments	References
Vesicular stomatitis virus (VSV-G)	HIV-1	Very wide tropism. Presents resistance to high-speed centrifugation.	[16][64][65][66][66][66][67 - 67][67 – 69]
	HIV-2		
	FIV	Cytotoxic for producer cells if expressed constitutively.	
	EIAV		
	SIV	Susceptible to complement-mediated degradation which can be minimized by PEGylation	
	BIV		
	JDV		
CAEV			
Feline endogenous retrovirus (RD114)	HIV-1	More efficient and less toxic than VSV-G in cells of the hematopoietic system	[70][71]
SIV			
Ebola	HIV-1	Efficiently transduces airway epithelium	[72]
Lymphocytic choriomeningitis virus (LCMV)	SIV	Low toxicity	[73]
	HIV-1		
	FIV		
Rabies	HIV-1	Rabies confers retrograde transport in neuronal axons	[24]
Mokola	EIAV	Mokola selectively transduces RPE upon subretinal injection	[24][74]
Ross River virus	HIV-1	Transduces hepatocytes, glia cells and neurons	[75][76]
FIV			
Sindbis virus	HIV-1	pH-dependentendosomal entry. Useful for vector targeting	[77]
Influenza virus hemagglutinin	HIV-1	Transduces airway epithelium	[72]
Moloney murine leukemia virus 4070 envelope	HIV-1	Able to transduce most cells	[18][16]
SIV			

Table 1. Lentiviral Vectors pseudotyped with various heterologous viral glycoproteins. Adapted from [78,79].

The envelope proteins engineered by fusion of natural ligands were in general able to bind to target cells. However the fusion domain of Env resulted generally in low vector titers since the ligand inhibits the fusogenic properties of the Env protein that allows for viral entry [85]. This approach seems to be more challenging but there are already improvements. One example is the LV pseudotyped with a chimeric glycoprotein of Sindbis virus covalently linked with mouse/human chimerical CD20-specific antibody which resulted in specific and stable transduction of CD20+ human lymphoid B cells. In this case the membrane fusion is triggered by the glycoprotein, in a pH-dependent manner, and it happens inside endocytic vesicles formed after antibody binding [86].

Other glycoproteins and ligands are being tested and used as well as alternative strategies to improve infection specificity of LVs [87–91].

4. Lentiviral vector production

The continuous research in LV development in the last twenty years and the acquired knowledge from the previous development of γ -RVs allowed the production of LVs with a significant biosafety level. However to apply LVs to clinical use they need to be easily and inexpensively produced and purified at a large-scale since, high concentrations of lentiviral particles are usually needed for efficient gene transfer in primary cells and the treatment of a single patient may require several liters of viral supernatant [92,93]

For large-scale and clinical-grade LV productions, a stable LV producer cell line seems to be the best approach for increased safety and well characterized production process. However, unlike γ -RVs, the development of LV packaging cell lines has been more challenging because of the cytotoxicity of some viral proteins like Tat, Nef, Vpr, Rev and PR [94]. Also VSV-G envelope, the typically envelope of choice for LV production because of its wide tropism and stability conferred to viral particles, is toxic for the producer cells. The VSV-G envelope can however be replaced by other non-toxic envelopes as the feline endogenous virus RD114 or the amphotropic MLV 4070A Env glycoproteins [33,95] and thus among the cytotoxic lentiviral proteins just the protease is still necessary for lentiviral vector production with the current packaging systems [93].

HIV protease mediates its toxicity *in vitro* and *in vivo* by cleaving procaspase 8, originating the casp8p41 fragment. This fragment induces mitochondrial depolarization leading to mitochondrial release of cytochrome C, activation of the downstream caspases 9 and 3 and nuclear fragmentation [96–98]. This cytotoxicity has hampered the development of stable cell lines.

The most used cells for LV production are the human embryonic kidney HEK-293 cell line and its genetic derivatives the 293T (expressing the SV40 large-T antigen) and 293E (expressing the Epstein-Barr virus nuclear antigen-1, EBNA-1) cell lines. For clinical application human 293 and 293T cells have been the exclusive cell substrates [93]. Both cell lines can be used to produce LV in adherent systems and both can be easily adapted to serum-free suspension cultures. The 293T cells are most widely used because presents superior LV productivities compared with HEK-293 cells. However the HEK-293 cell line may have an advantage in terms of safety as it lacks the SV40 large T antigen encoding gene (expressed in 293T cells) which is oncogenic [93,99,100]. In some research works other human or monkey derived cells have been used (other 293 derived clones, HeLa, HT1080, TE671, COS-1, COS-7, CV-1), although most of them showed lower LV titers [101]. However, COS-1 cells have shown to be capable of producing 3-4 times improved vector quality (expressed in infectious vector titer per ng of CA protein, p24), comparing with 293T cells, under serum-containing conditions [102].

4.1. Transient lentiviral vector production

Commonly LVs are produced by co-transfecting cells with the several expression cassettes harboring the transgene and the viral elements using chemical agents (e.g. calcium phos-

phate or polyethylenimine) and after 24 to 72 hours the LV are harvested [93]. This production system is fast and can be easily adapted to produce LVs with new genes of interest or with other Env glycoproteins. It is a simple process to apply at small scales commonly used in research, unlike the laborious development of a packaging cell line. However transient production is not the ideal choice for large and clinical LV productions since it is difficult to scale-up and requires large amounts of Good Manufacturing Practices (GMP) grade plasmid expressions cassettes turning the production more expensive [93,103]. In addition, transient LV production brings some biosafety problems like recombination between expression cassettes that could originate or facilitate the RCL formation. The recombination can occur in the mixture of transfection, inside the producer cells or during reverse transcription in the target cells since, generally after transfection cells have several copies of the plasmids which can contribute for the co-packaging of RNA transcripts [33,104]. Also batch to batch variability is common in transient productions since a population of transfected cells that expresses viral elements from episomal cassettes is generated. This can further complicate biosafety validation steps.

Nevertheless transient LV production is commonly used and recently it was shown that high titers of HIV-based LVs for clinical applications can be obtained by transient calcium phosphate transfection at large-scale under GMP conditions (Table 2) [99].

Cell origin	Vector	Packaging generation	Envelope	Maximal titers (I.P./ml)	Observations	Reference
293 E	SIN HIV-1 based	3 rd	VSV-G	1x10 ⁶	PEI-mediated transfection	[107]
HEK293	HIV-1 based	3 rd	VSV-G	1x10 ⁸	PEI-mediated transfection	[101]
293T	HIV-1 based	3 rd	VSV-G	2x10 ⁹	Transfection with calcium phosphate	[99]
293T	HIV-1 based	3 rd	VSV-G	1x10 ⁸	Transfection by Flow Electroporation	[105]

Table 2. Transient LV productions. In this table they are presented several features of recent lentiviral productions in a transient manner.

There are several transfection agents that can be used to transfect mammalian cells as calcium phosphate, polyethylenimine (PEI) and cationic molecules (such as LipofectAMINE® and FuGENE®). For large scale only Ca-phosphate and PEI are used since the others are much more expensive. Both reagents are efficient but PEI is usually preferred since Ca-phosphate efficiency is highly sensitive to pH variations and can require serum or albumin to reduce Ca-phosphate cytotoxicity, unlike PEI [93]. However their use can raise some purity problems and can be cost-ineffective. Recently a method that does not use chemicals for transfection, flow electroporation, was used for transiently LV production at

large-scale [105]. The electroporation systems are normally used to transfect small volumes but flow electroporation addresses this limitation by continuously passing the desired volume of a cell and DNA suspension between two electrodes [106]. The procedure can be effectively scaled up for large bioprocessing avoiding additional costs and purification problems (Table 2) [105].

4.2. Stable lentiviral vector production

To overcome the biosafety problems in LV transient productions, inducible packaging cells lines have been developed (Table 3). The development of these systems is more time-consuming since after insertion of each expression cassette the population of stably transfected cells is usually screened for the best producer clone, like for γ -RVs, to maximize the LV production. However, these packaging cell lines are derived from one clone, therefore all the cells have the same growth and LV production behavior being the LV productions reproducible. This allows the generation of GMP cell banks, increasing safety conditions.

Cell origin	Vector	Packaging generation	Envelope	Maximal titers (I.P./ml)	Observations	Reference
293T	HIV-1 based	2 nd	VSV-G	1x10 ⁷	Tet-off	[108]
293T	HIV-1 based	3 rd	VSV-G	1.8x10 ⁵	Ecdysone inducible system. Codon-optimized gag-pol	[109]
293T	SIV-based	3 rd	VSV-G	1x10 ⁵	Ponasterone inducible system. Codon-optimized gag-pol	[110]
293T	HIV-1 based	2 nd	VSV-G	3x10 ⁵	Tet-off. Codon-optimized gag-pol	[103]
293T	HIV-1 based	3 rd	VSV-G	3.4x10 ⁷	Tet-on	[111]
293T	EIAV based	3 rd	VSV-G	7.4x10 ⁵	Tet-on	[112]
293T	SIV-based	3 rd	VSV-G	5x10 ⁷	Introduction of vector by concatemeric array transfection. Tet-off	[113]
293T	HIV-1 based	2 nd	Ampho GaLV RDpro	1.2x10 ⁷ 1.6x10 ⁶ 8.5x10 ⁶	Continuous system. Codon-optimized gag-pol	[33]

Table 3. Lentiviral vector packaging cell lines. In this table they are presented several features of available packaging cell lines for LV production.

In conditional packaging cell lines the expression of cytotoxic proteins is under control of inducible promoters and the number of cells and growth conditions can be controlled, starting the LV production at a defined moment by adding an inductor or removing the suppressor from the culture medium. Originally the titers were low but further improvements in the expression cassettes design and optimization of the induction parameters led to similar levels of transient productions. However, such systems can only produce LV for a few days because of the activity of the cytotoxic viral proteins. In addition these packaging cells have often shown to be instable due to leaky expression of the cytotoxic viral elements that are under control of the inducible promoters and the need to add an inductor to the medium in some systems can add further difficulties to the purification process [93].

In 2003 Ikeda and co-workers have reported the development of a non-inducible packaging cell line that continuously produces LV for three months in culture (Table 3). However, significant titers could only be obtained after MLV-based vector transduction. This procedure raises serious problems from the biosafety point of view, since it increases the chances of RCL by homologous recombination, posing further concerns of co-packaging [37]. Nevertheless it was shown that it is possible to establish a cell line that can continuously produce LV although, until now no additional reports for this system appeared.

5. Lentiviral vector applications

Lentiviral vectors have emerged as powerful and versatile vectors for *ex vivo* and *in vivo* gene transfer into dividing and non-dividing cells. The particular characteristics of LVs allied to their marked development during the last years have triggered the attention of different fields, consequently a vast range of applications for these vectors, from fundamental biological research to human gene therapy have appeared. One of the applications of LVs is in genome-wide functional studies. The combination of synthetic siRNAs (small interfering RNA) or shRNAs (short hairpin RNAs) that can suppress the expression of genes of interest in mammalian cells [114], with engineered LVs allowed the formation of libraries like the Netherlands Cancer Institute (NKI) libraries, the RNAi consortium (TRC) libraries, the Hannon–Elledge libraries, and the System Biosciences (SystemBio) libraries for high-throughput loss-of-function screens in a wide range of mammalian cells [115]. For example, the TRC shRNA library has nearly 300,000 shRNAs targeting for 60,000 human and mouse genes [116]. The ability of LVs to achieve stable high-efficiency gene silencing in a wide variety of cells including primary cells, that are difficult to transduce, or non-dividing cells such as neurons thus greatly expanded the possibility of the RNAi screens [117].

Other application for LVs is in animal transgenesis. Genetic-modified animals can be created by infection of fertilized or unfertilized oocytes, single-cell embryos, early blastocysts, embryonic stem cells or by transduction of cells that are used as donors of nucleus for somatic cell nuclear transfer (SCNT) [10]. These animals (transgenic mice, rats, pigs, cows, chicken, monkeys) are used to understand gene function or biological processes, for validation of drug targets, for production of human therapeutic proteins and as preclinical models for human diseases [118].

Lentiviral vectors are being increasingly used for the cell genetic modification leading to cell-engineering applications. Stable gene transduction can be used for *in vivo* imaging of vector infected cells. *In vivo* imaging studies of cells, including stem cells, have become increasingly important to understand cell distribution, differentiation, migration, function, and transgene expression in animal models. As an example, LVs expressing the firefly luciferase gene were used to monitor human embryonic stem cell (hESC) engraftment and proliferation in live mice after transplantation [119]. LVs can also be used to cellular reprogramming of somatic cells. More specifically, the promising induced pluripotent stem cells (iPS) can be generated from a somatic cell by transduction of four key transcription factors, Oct4, Sox2, Klf4, and c-Myc, using LVs[120,121]. iPS can be used to study stem cell biology, as a cellular platform for pharmacological and toxicological [122] and are considered a possible source of autologous stem cells for use in regenerative medicine. LVs also have been used in biotechnology to engineer cell lines for the production of proteins of interest [123].

The main goal of LV technology is their use in clinical gene therapy applications. Within this purpose considerable efforts have been made to increase the safety and efficacy of LVs. Proof-of-concept has been established in preclinical animal models since several research groups have reported that LVs could treat or cure a disease including β -thalassaemia[124], sickle cell anemia [125], hemophilia B [126] and ζ -chain-associated protein kinase of 70 kDa immunodeficiency [127]. Moreover, improvements in other genetic disorders like Parkinson's disease [128], cystic fibrosis [129] and spinal muscular atrophy [130] have been reported.

LVs have more recently moved beyond the preclinical stage into the clinical arena. The first human clinical trial using LVs was initiated in 2003. In this, a VSV-G-pseudotyped HIV-based vector was engineered to conditionally express an antisense RNA against envelope glycoprotein in the presence of regulatory proteins provided by wild-type virus. Five subjects with chronic HIV infection received a single dose of gene-modified autologous CD4⁺ T cells which resulted in an increase of CD4⁺ T cells (in four out of the five subjects) and decrease in the viral load (in all five participants) after 1 year. Further studies over 2 years have not detected any adverse clinical events [131].

Since this first gene therapy clinical trial until June 2012, about 54 gene therapy clinical trial using LVs are ongoing or have been approved. Among them there are 12 trials for the treatment of HIV infection, 22 for the treatment of monogenic diseases (X linked cerebral adrenoleukodystrophy, Sickle cell anemia, Wiskott-Aldrich Syndrome, Metachromatic Leukodystrophy, X-Linked Chronic Granulomatous Disease, Inherited Skin Disease Netherton Syndrome, mucopolysaccharidosis type VII, β -thalassemia, Fanconi Anemia Complementation Group A, X-Linked Severe Combined Deficiency, Adenosine Deaminase Deficient Severe Combined Immunodeficiency, Hemophilia A), 15 against various cancers, 2 for Parkinson's disease, 3 for ocular diseases and 1 for patients with Stargardt Macular Degeneration [1].

6. Conclusions and outlook

The major concerns associated with the use of all retroviral vectors are the formation of replication competent retroviral vectors (RCR), the mutational integration of the provirus into the host cellular genome and mobilization of structural viral genes to target cells. In addition, the majority of developed LVs are HIV-derived raising further safety concerns since this is a well known human pathogen. Significant efforts have been made to develop LVs with improved biosafety and increased transduction efficiency. Some of those biosafety features include the splitting of viral elements by several expression cassettes, the use of self-inactivating vectors (SIN), decreasing to a minimum the number of viral elements and reducing homology between them.

Lentiviral vectors have already won its place as valuable and flexible tool for gene delivery, being used in several applications but further research is still ongoing towards the development of a lentiviral vector providing higher titers, higher robustness, lower toxicity and higher biosafety.

Lentiviral vector gene therapy is becoming a real alternative vector for therapy with dozens of clinical trials either been already performed or ongoing. These, together with the future incoming clinical trials, will enable to assess overall the pros and cons of the newcomer lentiviral vectors and will provide insights to further vector innovations that will be important to increase their productivity, quality and safety.

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Lentiviral Vectors in Immunotherapy

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Additional information is available at the end of the chapter

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1. Introduction

Genetic immunotherapy can be defined as a therapeutic approach in which therapeutic genes are introduced into defined target cell types to modulate immune responses. A major challenge for this therapeutic strategy is the delivery of these genes into target cells in an efficient, stable manner. Possibly one of the best systems to achieve this is the use of lentiviral vectors (lentivectors) as gene carriers, as they are capable of transducing both dividing and resting cells [1].

Lentivectors are mainly derived from the human immunodeficiency virus (HIV-1) genome, a member of the *Retroviridae* family. The defining characteristic of retroviruses is their capacity to stably integrate their RNA genome into the host cell chromosomes, in the form of a cDNA copy (Figure 1). Therefore, retrovirus and lentivirus vectors have been used extensively in research since they are ideal gene carriers into target cells. Moreover, both retrovirus and lentivirus vectors have been successfully applied in human gene therapy for the treatment of several genetic/metabolic inherited diseases (Cartier et al, 2009; Cavazzana-Calvo et al, 2010; Gaspar et al, 2004; Grez et al, 2010; Ott et al, 2006; Thrasher et al, 2006).

Lentiviruses are spherical enveloped viruses with a diameter around 80 to 120 nm and contain two copies of a single-stranded RNA genome (Figure 2) [2]. The genome is enclosed within a core composed of the structural and enzymatic proteins nucleocapsid (NC), capsid (CA), reverse transcriptase (RT), integrase (IN) and protease (PR). The core is surrounded by a protein layer of matrix (MA) protein. The envelope protein (ENV) is embedded in the virion lipid envelope, and it binds to the target cellular receptor and mediates virion entry.

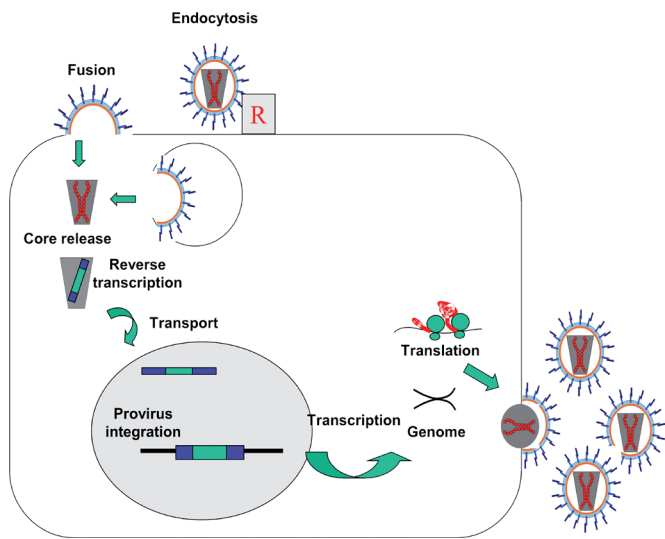


Figure 1. The retrovirus life cycle. The life cycle of retroviruses, including lentiviruses is shown in this figure as a multi-step mechanism, starting with virion binding to the cellular receptor (R), leading to direct fusion or endocytosis. Then, the internal core is released and the two RNA molecules undergo reverse transcription as indicated, ending up with a single cDNA molecule. The core is then transported to the nucleus (in the case of lentiviruses) and the cDNA is integrated into the cell chromosome. The integrated genome (provirus) undergoes transcription, producing more RNA genome copies (and also spliced mRNAs, not shown here), which are also translated into structural and enzymatic proteins. These are then assembled into virions that bud out of the infected cells.

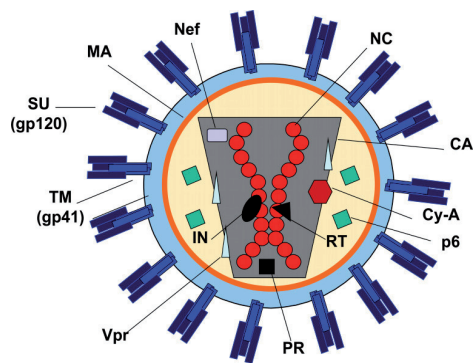


Figure 2. In this scheme, the lentivirus virion is represented as a sphere containing a genome made of two RNA molecules associated to the nucleocapsid (NC) protein. The nucleocapsid is enclosed by a core made of capsid (CA) protein, which is surrounded by a shell of matrix (MA) protein that associates to the virion envelope. The two subunits of the HIV-1 envelope are also indicated (SU and TM). In addition, other enzymatic (IN, RT, PR), accessory (Vpr, p6) and cellular (Cyclophilin A, Cy-A) proteins are shown, which are incorporated into lentivirus particles.

Lentivectors are classified as complex retroviruses according to their genome organisation, as it contains accessory and regulatory genes absent in other retroviruses. Nevertheless, the retrovirus genome shares a common 5' to the 3' gene organisation, with Gag, Pol and Env genes [1, 3]. Gag encodes MA, CA and NC as a polyprotein. Pol encodes enzymatic proteins associated to reverse transcription, that is, the reverse transcriptase (RT), integrase (IN) and protease (PR). RT synthesizes a single cDNA copy from the retrovirus genomic RNA [4]. IN mediates cDNA integration in the host cell chromosome, while PR cleaves Gag and Gag-Pol polyproteins during virion maturation.

The integrated cDNA genome is flanked by two long terminal repeats (LTRs) subdivided in U3, R and U5 regions. U3 is the HIV-1 promoter. The R region marks the starting point of transcription, and U5 region is critical for reverse transcription. The other key elements are the packaging signal (Ψ) and the polypurine tract (PPT). The packaging signal, as in many other virus species, allows RNA genome encapsidation during virion assembly in the cytoplasm. The PPT element is a key element for reverse transcription [5].

Lentivectors are usually obtained following a three-plasmid co-transfection in 293T cells (Figure 3) [6, 7]. The first one, the packaging plasmid, provides the structural and RT proteins (Gag-Pol). The second one, the envelope plasmid, encodes a glycoprotein to pseudotype the lentivector particles. This process consists on the incorporation of an heterologous Env in the viral lipid envelope. This will allow the lentivector to exhibit the specific cell tropism given by the Env used in pseudotyping. One of the most used Env is the Vesicular Stomatitis Virus (VSV) Glycoprotein (G). The VSV-G confers stability to the lentivector particles and a very broad tropism for human and non-human cells [8]. Lastly, the third one, the transfer plasmid, contains the cis-acting sequences for replication/transcription and packaging (Figure 3) [9]. By including promoters within the transfer plasmid, any gene of interest can be expressed either constitutively or inducibly, in a cell type-specific or unspecific manner (Figure 3) [1]. Therefore, lentiviral vectors can also incorporate genes with immunoregulatory properties in cells from the immune system.

Two main cell types of the immune system have been preferential targets for genetic immunotherapy: antigen presenting cells (APCs) and effector T lymphocytes. These two cell types are key controllers of immune responses. By expressing transgenes of interest in APCs, such as DCs, they can be processed and presented to antigen-specific T cells in the immunological synapse. This antigen presentation is the first step in either starting of suppressing immune responses. Therefore, if genes with modulating properties of APC functions can be co-expressed with antigens, the strength and type of immune response can be controlled. In fact, genetic modification of cells from the immune system can circumvent the limitations of current immunotherapeutic protocols. Using targeted lentiviral vectors, specificity and effectiveness can be achieved by targeting key cells that modulate and polarize immune responses.

Although more challenging than DCs, T lymphocytes can also be genetically modified using lentiviral vectors. Vectors expressing T cell receptors (TCRs) specific for antigens of interest can modify the specificity of T cell populations, or expand their antigen profile. Therefore, these genetically modified T cells can be adoptively transferred in the human patients. This

strategy is particularly important to generate T cells with high affinity TCRs towards tumour-associated antigens.

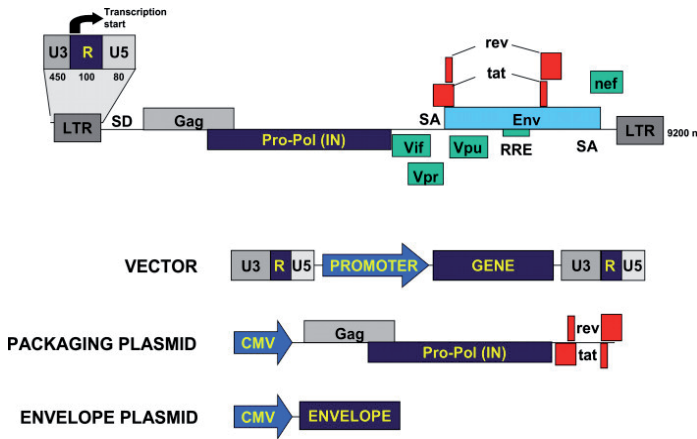


Figure 3. The HIV-1 genome is shown at the top of the figure. All structural, accessory and enzymatic genes are indicated throughout the genome. The two LTRs are shown as present in an integrated provirus. The three functional regions of the HIV LTR are shown on top of the 5'-LTR. Numbers indicate nucleotide positions. The HIV genome is splitted in three different plasmids to engineer a gene vector. The transfer (vector) plasmid is indicated, with only the HIV-1 LTRs containing an internal promoter driving the expression of a gene of interest. In the packaging plasmid, only the Gag-Pro-Pol and Rev, Tat genes have been retained. This increases biosafety. Transcription of these genes takes place under the control of the cytomegalovirus promoter (CMV), as indicated in the figure. Lastly, a third plasmid encoding an envelope glycoprotein is shown on the bottom of the figure. This envelope pseudotypes the lentivector particle.

2. Genetic modification of DCs with lentivectors

For the elimination of cancer cells and chronic infections such as HIV, hepatitis B and malaria, a strong, effective T cell response is required. To initiate these strong T cell responses, the interaction between antigen-specific T cells and antigen-presenting APCs has to be strengthened[10]. Amongst APCs, DCs are most frequently the targets of immunotherapy protocols since they are probably the most immunogenic [10, 11].

To activate T cells during antigen presentation, these T cells have to receive at least three different signals from APCs (Figure 4) [10, 12-16]. The first signal, or signal 1, is the direct recognition of the peptide-major histocompatibility molecule complex (p-MHC) by the TCR. However, this interaction is not sufficient to confer T cells with effector activities. For this, a second co-stimulatory signal (signal 2) has to be co-delivered together with p-MHC recognition. This signal 2 is the consequence of the integration of activatory and inhibitory interactions between ligands/receptors on the surface of DCs and T cells (Figure 4)[16, 17]. For example, CD80 binding to CD28 is strongly activatory, while CD80 binding with CTLA-4, or

PD-L1 with PD-1, are strongly inhibitory[16, 17]. Apart from these two signals, T cells require a third signal which drives their differentiation into distinct subtypes that will regulate different types of immune responses [13, 18]. This third signal is usually provided by different combination of cytokines present within the immunological synapse (Figure 4). For example, the presence of high levels of IL12 will polarise T cell differentiation towards a Th1 type (cellular cytotoxic immunity). On the other hand, high levels of IL10 will drive polarisation towards Th2 (antibody responses).

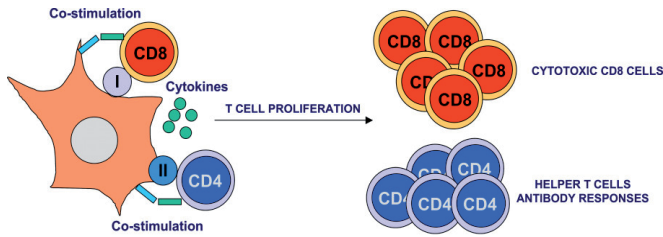


Figure 4. In this scheme, DCs (left) present antigens to specific CD8 and CD4 T cells (as indicated) in the context of MHC class I (I) or class II (II) molecules. These T cells receive further stimuli by co-stimulation through ligand-receptor interactions between the DC and T cells (as indicated in the figure). Simultaneously, activated DCs secrete cytokines and chemokines (indicated in the figure as spheres) that will drive T cell activation, proliferation and differentiation into either cytotoxic CD8 T cells or T helper cells, as shown on the right.

An ideal immunotherapeutic approach would be to use lentiviral vectors to deliver the antigen of interest together with the three signals required for the desired T cell polarisation. Lentivectors have been extensively used for this purpose, because they are particularly effective in transducing DCs without affecting their functionality, unlike other vectors such as those based on adenoviruses [8, 19-26]. In fact, the stable integration of the lentivector genome allows long-term, sustained transgene expression[1, 9]. In addition, the expressed transgene is processed and its antigen peptides loaded in MHC I and MHC II molecules [27]. This is of the outmost importance for immunotherapy, since expressed proteins can be processed and loaded onto MHC-II molecules through several pathways. While secreted proteins can enter the endocytic pathway and membrane proteins can be recycled towards the endosomal pathway, cytoplasmic proteins can still enter the MHC II pathway by autophagy[28]. Nevertheless, to improve MHC II loading of peptides from cytoplasmic proteins, endocytic localisation sequences can be fused to the transgene, with the lysosomal-associated membrane protein 1 or with the amino-terminal portion of the MHC II invariant chain [29-32].

The use of lentivectors to express whole transgenes rather than antigen peptides circumvents the necessity of designing specific peptide/protein vaccines for loading into specific MHC genotypes [27]. Thus, lentivectors expressing model antigens have been extensively used as a proof of principle. Amongst others, the antigens chicken ovalbumin (OVA), tumour-tumour associated antigens such as MELAN-A, tyrosinase related protein (Trp), NY-ESO or antigens from infectious agents have been expressed in DCs. These modified DCs induced strong activation and proliferation of antigen-specific T cells. [17, 33-38].

3. Lentivector immunogenicity

Lentivectors have been extensively used in vaccination protocols, due to their capacity of raising strong transgene-specific immune responses [9, 17, 30, 38-40]. Interestingly, some reports suggest that lentivectors are incapable of inducing DC maturation *in vitro*, suggesting that some components of the lentivector preparations provide signals 2 and 3 through a mechanisms not well understood [40, 41].

DC maturation is a complex, step-wise process in which they up-regulate the surface expression of co-stimulatory molecules such as CD80, CD83, CD86, CD40, adhesion molecules such as ICAM-1 and also the expression of MHC molecules. In general terms, DC maturation can be triggered by recognition of pathogen-derived molecules by specific receptors on the DC surface, such as the family of toll-like receptors (TLRs) [42, 43]. DCs can also mature through the exposure of pro-inflammatory cytokines by a process called cytokine priming [13-15]. Matured DCs can effectively provide strong signals 1 and 2, leading to efficient T cell activation and proliferation. Thus, their administration *in vivo* induces DC maturation and production of type I interferon that can provide signal 3 [9, 39, 44, 45].

The capacity of lentivectors to induce DC maturation after vaccination is probably caused by either specific components of the lentivector particle or by contaminants present in the lentivector preparation. As a matter of fact, lentivector particles resemble viruses and therefore, some components have the potential to stimulate immune responses such as the RNA genome or the cDNA [46]. These are ligands for TLR7 and TLR9, respectively [41, 45]. In addition to specific components of the lentivector particle, contaminants can also alter their immunostimulatory properties. In fact, most lentivector preparations pseudotyped with VSV G contain VSV-G tubulo-vesicular structures enclosing plasmid DNA that stimulate TLR9 *in vitro*, leading to type I IFN production by pDCs [47]. In addition, foetal calf serum (FCS) contributes to immunogenicity by providing T cell epitopes with adjuvant capacities [48].

4. Control of DC maturation by expression of molecular activators with lentivectors

Lentivector preparations can induce DC maturation *in vivo*. However, in some circumstances this is not enough for effective therapeutic activities. This is the case for cancer immunotherapy, in which breaking tolerance towards TAAs is still a medical challenge. One possible solution is to co-express TAAs with molecular activators of DCs using lentivectors, particularly using activators of signalling cascades belonging to the TLR pathways.

This has been firstly achieved by over-expressing adaptor molecules, which associate with TLR cytoplasmic tails. These adaptor molecules recruit activatory protein kinases leading to DC maturation. Thus, lentivectors have been used to express MYD88 or TRIF1 in mouse myeloid DCs, which also increases secretion of pro-inflammatory cytokines IL-6, IL-12 and IFN- α , which enhanced T cell cytotoxicity [49]. The NF- κ B pathway has also been an attractive target because it controls transcription of the majority of pro-inflammatory genes (Fig-

ure 5). Lentivectors have thus been used to express the Kaposi's sarcoma-associated herpes virus FLICE-like inhibitory protein (vFLIP), a constitutive activator of NF- κ B by direct association and activation of NF- κ B essential modulator (NEMO) [50-53]. In fact, vFLIP expression has resulted to be a strong adjuvant when expressed in DCs, leading to strong DC maturation and effective CD4 and CD8 T cell responses. Lentivector expression of vFLIP significantly improves anti-tumour activities in a lymphoma mouse model and anti-parasitic efficacy in an OVA-expressing leishmania model [38, 54]. Lentivectors have also been used to inhibit negative regulators of NF- κ B activation, such as the ubiquitin ligase A20. Lentivectors have successfully delivered to DCs short hairpin RNAs (shRNAs) targeting A20. The abrogation of A20 expression caused DC maturation, effective CD8 cell responses and inhibition of regulatory T cells (Tregs) [55, 56].

Other molecular activators of mitogen activated protein kinases (MAPKs), activated after TLR engagement, have also been co-expressed in DCs with antigens of interest (Figure 5). MAPKs are mainly divided in three groups, ERK, p38 and JNK. While ERK is associated to survival and immune suppression, p38 and JNK are thought to stimulate DC maturation and inflammation (Figure 5). Constitutive p38 activation was achieved by expressing the MKK6 EE mutant using lentivectors, and it resulted in CD80, CD40 and ICAM-I up-regulation without significant secretion of pro-inflammatory cytokines [17, 40, 41]. A similar result was achieved by JNK1 activation, following expression of the MKK7-JNK1 fusion gene in DCs. Interestingly, although a full DC maturation phenotype was not achieved *ex vivo*, co-expression of these MAPK activators with an OVA-containing transgene or MELAN-A induced significant antigen-specific CD4 and CD8 T cell responses. Moreover, these lentivectors improved survival in a murine tumour model for lymphoma, both with integrating and non-integrating lentivectors [38].

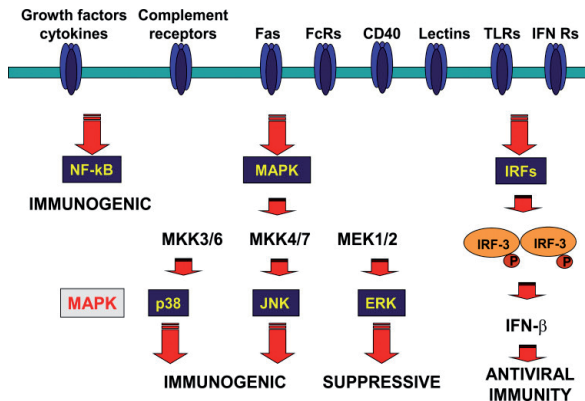


Figure 5. Intracellular signalling pathways regulating DC functions.

Other molecules have been applied for DC maturation. For example, CD40 ligand expression achieved human DC activation and up-regulated the expression of CD83, CD80, MHC-I

and induced IL-12 secretion [57]. This strategy increased CD4 and CD8 responses towards influenza epitopes and the TAA gp100. Co-expression of DC-promoting cytokines such as GM-CSF- and IL-4 using lentivectors resulted in long-lasting immunity against melanoma when co-expressed with TAAs Trp-2 and Mart-1 [58].

In this scheme, the main signalling pathways triggered after the engagement of a wide range of receptors on the DC surface (see the indicated receptors embedded in the membrane) with their ligands. Engagement of these receptors starts a complicated cascade of signalling pathways that will converge in a few, well-characterised ones, the NK- κ B, MAPKs and interferon regulatory factors (IRFs) (as indicated below the membrane). Some of these pathways, such as NK- κ B, MAPKs p38 and JNK1 are pro-inflammatory and lead to DC maturation. Others, such as ERK, are clearly immunosuppressive.

5. Control of DC maturation by inhibiting negative co-stimulation using lentivectors

DC maturation can also up-regulate molecules that provide negative stimulation to T cells, such as programmed cell death receptor ligand 1 (PD-L1) and PD-L2, the ligands for the PD-1 receptor on T cells. Negative co-stimulation is part of a regulatory mechanism that controls the activation state of T cells following antigen presentation [17, 59, 60]. Thus, interference with negative co-stimulation could in principle reinforce T cell activation and enhance cytotoxic activities. Therefore, lentivectors have been used to deliver shRNAs in DC against PD-L1. PD-L1 silencing in antigen-presenting DCs hyperactivated T cells by preventing the up-regulation of Casitas B-lymphoma (Cbl)-b E3 ubiquitin ligase. This strategy co-accelerated anti-tumour immune responses, particularly if combined with a p38 activator or dominant negative mutant of MEK1, the upstream kinase of ERK [17, 59].

6. Lentivectors and cancer immunotherapy

Lentivectors are particularly promising in cancer immunotherapy, for which conventional immunization is largely ineffective due to two major barriers. Firstly, TAAs are generally self-proteins to which there is strong immunological tolerance. Secondly, that tumours are strongly immune-suppressive and they use several mechanisms to avoid immune responses [41].

Lentivectors can be used in cancer immunotherapy in two different ways. In the first one, DCs can be generated *ex vivo* from the patient, followed by lentivector transduction and *in vivo* administration. Thus, cellular vaccination with transduced DCs expressing HLA-Cw3 induced activation and proliferation of CD8 T cells in a mouse model [37]. Similarly, lentivector transduction was shown to be superior to peptide pulsing in inducing OVA-specific T cell responses [61], protected mice from OVA-expressing tumour cells and significantly inhibited tumour growth. The second strategy is direct lentivector vaccination, taking advantage of their intrinsic immuno-stimulatory capacities and their reduced cost [24, 26, 35].

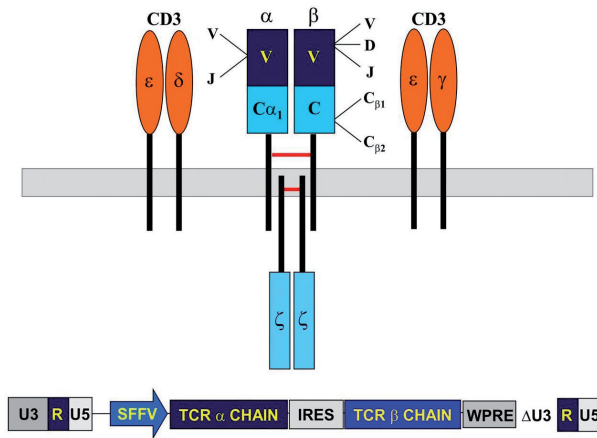


Figure 6. A scheme of the TCR is shown embedded in the cellular membrane. Both α and β chains are shown as indicated, subdivided in variable and constant regions (V, C). The other CD3 chains that associate with the TCR are also included in the figure. On the bottom, a lentivector co-expressing α and β TCR chains is shown, under the control of the spleen focus forming virus promoter (SFFV) and an internal ribosome entry site (IRES) [67]. This particular lentivector is self-inactivating (SIN) and presents a deletion of viral enhancers in the 3' LTR. When this construct is integrated, the 5' LTR disappears and it is replaced with the deleted version.

As mentioned above, a major issue with cancer immunotherapy is that most TAA-specific T cells may have been eliminated during thymic clonal deletion. Thus, even if effective and strong DC maturation is achieved, no effective responses will be achieved due to lack of TAA-specific T cells. To circumvent this, TAA-specific T cells can be generated by lentivector transduction *in vitro*, and adoptively transferred in patients (Figure 6) [62]. Clinical efficacy has been reported for melanoma, synovial cell sarcoma, colorectal, neuroblastoma and lymphoma, but using γ -retrovirus vectors instead of lentivectors [63-66].

T cells are largely refractory to transduction by VSV G-pseudotyped lentivectors, and they require some level of T cell stimulation [67]. Treatment with IL-2 and IL-7 allows lentivector transduction and preserves a functional T cell repertoire [68, 69]. As an example, Wilms tumour antigen (WT1)-specific T cells were generated by lentivector expression of a WT1-specific TCR in the presence of IL-15 and IL-21. These modified T cells were multifunctional and exhibited the expected antigen specificity [67]. This approach of T cell modification is rather promising. In a clinical trial with 15 terminally sick melanoma patients, 2 showed complete regression and long-term survival after transfer of T cells expressing a MART-1-specific TCR using γ -retrovirus vectors [70]. Interestingly, it has been recently demonstrated that entivectors pseudotyped with measles virus H/F glycoproteins effectively transduce quiescent adult T cells in the absence of any exogenous stimulus, whether cytokines or anti-CD3/anti-CD28 stimulation. In fact, transduction with these lentivectors did not affect T cells in any way [17, 71-73].

7. Lentivector gene immunotherapy for the treatment of autoimmune disorders

It is relatively “straightforward” to achieve immune stimulation using lentivectors. However, the induction of immune suppression or tolerance with lentivectors is rather challenging. Nevertheless, the induction and maintenance of immunological tolerance is critical for homeostasis. The organism is permanently and closely in contact with a very wide range of antigens of many origins. A large majority of them are innocuous and do not pose a direct threat. Thus, the immune system must not respond to these antigens, as an immune response is associated with significant collateral tissue damage. The immune system should be activated only if a real threat appears. Therefore, the immune system possesses several tolerogenic mechanisms in place to keep immunological homeostasis. As mentioned before, a key one is clonal deletion of auto-reactive T cells in the thymus [74]. However, there is a significant number of auto-reactive T cells that escape from clonal deletion. Many of them will differentiate towards natural Foxp3 CD4 regulatory T cells [74-77].

In addition to clonal deletion and differentiation of natural Tregs, there are a number of tolerogenic mechanisms in place that regulate immune responses towards peripheral antigens. The organism is in permanent direct contact with many substances and commensal organisms in mucosal areas and in peripheral tissues. In these situations, inducible Tregs differentiate from naïve CD4 T cells after antigen presentation by tolerogenic DCs. These regulatory T cell types are usually classified in Tr1 (CD4 CD25 IL10 or TGF- β) and Th3 (CD4 CD25 Foxp3) cells [78-82]. Therefore, DCs can also be converted in tolerogenic by expression of immunomodulatory genes with lentivectors. This strategy opens up the application of lentivectors for the treatment of autoimmune disorders.

8. Induction of tolerogenic DCs using lentivectors

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9. Induction of tolerogenic DCs using lentivectors

DCs can induce immunological tolerance through a number of mechanisms. It is generally accepted that antigen presentation by immature DCs is poorly immunogenic, and results in Treg differentiation, T cell apoptosis and T cell anergy [83-86]. These immature tolerogenic DCs express low levels of co-stimulatory molecules CD80, CD86, CD83, CD40 and MHC molecules [10, 40, 41, 78, 87]. Resident mucosal DCs are intrinsically tolerogenic independently on their maturation phenotype as a consequence of the presence of retinoic-acid [88]. In addition, these DCs become strongly immunosuppressive due to contact with TLR agonists from commensal microbiota [88-90]. DCs can also become strongly immunosuppressive after treatment with lectin ligands or exposure to immunosuppressive cytokines such as IL-10, IL-4 or TGF- β [78, 87, 89-92]. Tolerogenic DCs usually express high levels of these immunosuppressive cytokines, even if they are phenotypically mature [10, 40, 78, 87, 89-93]. In this situation, they provide strong signals 1 and 2 to T cells, together with a simultaneous strong tolerogenic signal 3. For example, in the presence of bioactive TGF- β , strong antigen presentation leads to differentiation to antigen-specific Foxp3 Tregs, while secretion of IL-10 usually results in Tr1 differentiation [91, 93-95].

Tolerogenic DCs can also up-regulate molecules that provide an inhibitory signal to T cells, such as PD-L1 (or B7-H1), a member of the B7 co-stimulatory molecules [17, 96]. PD-L1 expression in DCs regulates T cell activities during antigen presentation and prevents T cell hyperactivation [17]. In addition, PD-L1-CD80 binding on T cells induces antigen-specific Treg differentiation [97]. Other members of the B7 family are immunosuppressive [98]. Immunosuppressive DCs also up-regulate aminoacid-metabolising enzymes, such as arginase or indoleamine 2,3-dioxygenase (IDO) [99-104]. It is thought that these enzymes deplete T cells of essential aminoacids.

Lentivectors can be used to confer tolerogenic activities to DCs by expression of immunoregulatory genes together with antigens of interest. The first strategy that was tested experimental was the expression of potent immunosuppressive cytokines. This approach was used with γ -retroviral vectors for inflammatory diseases [95, 105, 106], [105, 106]. Lentivectors have been applied in an experimental model of asthma by expressing IL-10, leading to expansion of IL-10-expressing Foxp3 Tregs with potent anti-inflammatory properties [107]. Alternatively, small immunosuppressive peptides can also be delivered with lentivectors, such as the vasointestinal peptide (VIP). Intraperitoneal administration of VIP-encoding lentivec-

tors in mice effectively inhibited the development of experimental collagen-induced arthritis. This was achieved by a markedly reduction of pro-inflammatory cytokine secretion and the expansion of Foxp3 Tregs [108]. The administration of genetically modified VIP-expressing DCs also showed significant therapeutic effects in EAE and in the coecal ligation and puncture (CLP) model [109].

DCs can also be reprogrammed by direct modulation tolerogenic signalling pathways within DCs (Figure 5). Therefore, lentivector expression of a constitutively active MEK1 mutant resulted in sustained MAPK ERK phosphorylation, resulting in immunological tolerance [40, 90, 110–114]. These genetically modified DCs exhibit an immature phenotype with low levels of CD40 and secretion of bioactive TGF- β [40, 78]. Antigen presentation by these ERK-activated DCs differentiated antigen-specific Foxp3 Tregs both *ex vivo* and *in vivo* in a mouse model [78]. Direct lentivector vaccination encoding the ERK activator effectively controlled antigen-induced inflammatory arthritis in a mouse model [78].

Similarly, lentivector expression of a constitutively active IRF3 mutant induced high expression levels of IL-10, and expanded antigen-specific Foxp3 Tregs which inhibited immune responses (Figure 5) [40]. Activation of endogenous negative feedback mechanisms of DC maturation pathways has also been applied to induce immune suppression. In this way, by over-expressing the suppressor of cytokine signalling 3 (SOCS-3) in DCs, pro-inflammatory signalling pathways were severely impaired [115]. These genetically modified DCs significantly decreased secretion of pro-inflammatory cytokines IFN- γ , IL-12 and IL-23, and showed an enhanced IL-10 production, which effectively inhibited experimental autoimmune encephalomyelitis (EAE) in mice [115].

An alternative strategy to generate tolerogenic DCs is the inhibition of pro-inflammatory signalling pathways instead of activating immunosuppressive pathways. As NF- κ B is a critical inflammatory signalling pathway, its inhibition is promising for the induction of immunological tolerance [41]. To achieve this, Rel-B was silenced by the delivery of a shRNA targeted to Rel-B [116]. In this way, its inhibition could effectively prevent DC maturation after engagement with TLR ligands, and it was sufficient to treat autoimmune myasthenia gravis in a mouse model [116]. In an analogous manner, lentivectors have also been applied to silence B cell activating factor (BAFF) in the inflamed joint [117, 118], which was very effective for the treatment of experimental collagen-induced arthritis [119] without the need of targeting the arthritogenic antigen. These lentivectors were directly injected in the inflamed joint, where they preferentially transduced resident DCs. BAFF silencing in these DCs inhibited their maturation, and most importantly, inhibited differentiation of pathogenic Th17 [119].

10. Conclusions

Classical immunotherapeutic strategies for the treatment of cancer and infectious diseases rely on either administration of the antigen peptides together with adjuvants, or the inoculation with attenuated strains of pathogenic agents. This approach has been largely successful for the treatment of a wide range of infectious agents. However, for cancer immunotherapy,

most potent and targeted immunotherapeutic approaches are required to break the natural tolerance towards TAAs. The targeted co-delivery of immunomodulatory genes with antigens of interests to DCs has opened the application of gene therapy for immunotherapy. Lentivectors exhibit a remarkable transduction capacity of DCs and also T cells, and thus, they are ideal tools to achieve immunodulation. In this way, the immune system can be strongly and specifically activated for the treatment of cancer and infectious diseases, but it can on the other hand be strongly immunosuppressed. This makes it possible the induction of immunological tolerance and treatment of autoimmune disorders.

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Targeted Lentiviral Vectors: Current Applications and Future Potential

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Additional information is available at the end of the chapter

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1. Introduction

About two decades ago recombinant human immunodeficiency virus type 1 (HIV-1) was proposed as a blueprint for the development of lentiviral vectors (LVs) (Naldini, Blomer et al. 1996). Lentiviral vectors exhibit several characteristics that make them favorable tools for gene therapy, including sustained gene delivery through vector integration, transduction of both dividing and non-dividing cells, applicability to different target cell types, absence of expression of viral proteins after transduction, delivery of complex genetic elements, low genotoxicity and the relative ease of vector manipulation and production (Cattoglio, Facchini et al. 2007; Bauer, Dao et al. 2008). This is reflected in the numerous applications such as: transgene (tg) overexpression (Lopez-Ornelas, Mejia-Castillo et al. 2011), persistent gene silencing (Wang, Hu et al. 2012), immunization (Breckpot, Emeagi et al. 2008), generation of transgenic animals (Baup, Fraga et al. 2010), *in vivo* imaging (Roet, Eggers et al. 2012), induction of pluripotent cells, stem cell modification (Sanchez-Danes, Consiglio et al. 2012), lineage tracking and site-directed gene editing (Lombardo, Genovese et al. 2007) as well as many applications targeting cancer cells (Petrigliano, Virk et al. 2009).

Recombinant LVs can be derived from primate as well as non-primate lentiviruses such as HIV-1 and simian immunodeficiency virus (SIV) next to the equine infectious anemia virus, caprine arthritis-encephalitis virus, maedi-visna virus, feline immunodeficiency virus (FIV) and bovine immunodeficiency virus respectively (Escors and Breckpot 2010). They are all members of the *Retroviridae* family with 'retro' referring to their capacity to retro-transcribe their diploid single stranded (ss) RNA genome into a double stranded (ds) DNA copy that is

integrated in the genome of the infected host cell (Figure 1A). Since LVs are most often derived from HIV-1, the generation of recombinant LVs has been accompanied by several safety concerns such as the generation of replication-competent lentiviruses (RCLs). Another potential biosafety concern is the induction of insertional mutagenesis, a major genotoxic problem that emerged in gene therapy clinical trials using their γ -retroviral counterparts (Manilla, Rebello et al. 2005). Generally, LVs are produced by transiently transfecting HEK 293 or 293T cells with plasmids encoding structural and functional sequences, imperative for proper LV particle generation. Over the last decades, vector development has largely focused on the design of these plasmids. Firstly, only critical viral structural and functional sequences are provided and secondly, these sequences are divided over a certain number of individual plasmids either in *cis* (encoded by the LV) or *trans* (packaged as a protein within the LV particle), with a minimal overlap between viral sequences. This led to a LV production procedure where at least three different plasmids are used: (1) a packaging plasmid which provides all viral structural and enzymatic sequences (encoded by *gag* and *pol*) in *trans* to generate a functional particle, (2) a transfer plasmid providing the expression cassette in *cis*, cloned into the non-coding remains of the original lentiviral genome (Figure 1B, adapted from (Delenda 2004)) including a packaging signal and the two long terminal repeats (LTRs) of which the promoter activity has been deleted from the 3' LTR and (3) an envelope plasmid encoding an envelope glycoprotein (gp) consisting of a transmembranary domain (TM) and a receptor-binding domain (SU) that determines the LVs' tropism (Figure 1A).

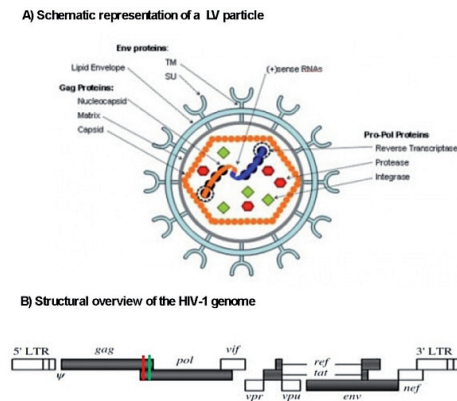


Figure 1. Schematic representation of an HIV-1 particle (A) and its genome (B). The diploid ssRNA genome of HIV-1 is stabilized by structural nucleocapsid proteins and together with the enzymatic proteins reverse transcriptase, protease and integrase packaged in a nucleocapsid structure, which in turn is enclosed by capsid proteins. This nucleocapsid is surrounded by a matrix protein layer and a producer cell derived phospholipid bilayer in which the envelope proteins consisting of an SU and TM part, are embedded (A). All proviral genes (*gag*, *pol*, *pro*, *vif*, *vpr*, *vpu*, *ref*, *tat*, *env* en *nef*) are flanked by two identical LTRs that consist of three regions: U3, R and U5. Within the U3 region, all proviral transcriptional control elements are situated such as the promoter and several enhancer sequences. Ψ represents the packaging signal. At the 3' end of the *pol* gene the central polypurine tract (red) and central termination sequence (green) are located. Both ensure the formation of a triple stranded DNA flap, crucial for nuclear entry of the pre-integration complex in non-dividing cells (B).

Besides this division over different plasmids, other important construct optimization steps have been implemented. While in the first generation LV packaging plasmids the entire *gag* and *pol* genes were encoded together with all accessory regulatory and virulence genes, the second generation was multiply attenuated by removal of the four virulence genes, but not the regulatory genes *tat* and *rev* (Zufferey, Nagy *et al.* 1997). In the third generation, the *rev* gene is expressed from a separate plasmid and the *tat* gene is removed by insertion of a strong constitutive promoter replacing the U3 region in the 5' LTR of the transfer plasmid (Dull, Zufferey *et al.* 1998). A major improvement was achieved with the development of SIN or self-inactivating LVs where a deletion in the U3 region of the 3' LTR of the transfer plasmid abolished the production of full-length vector RNA in transduced cells. This not only minimizes the risk for RCLs, but also reduces the chance that the viral LTR enhancers interfere with the expression cassette, which minimizes aberrant expression of adjacent cellular coding regions. Subsequently these and many other optimization steps paved the way towards a more effective and safer version of the lentiviral gene delivery vehicle (Romano, Claudio *et al.* 2003).

In addition to packaging and transfer plasmid optimization, also the envelope plasmid was modified by replacing the natural HIV-1 envelope gp with an alternative gp gene, most often the gp of vesicular stomatitis virus (VSV.G). This concept is called pseudotyping and VSV.G endowed the LV particle with an increased stability and broad cellular tropism (to most if not all mammalian cells). However, it became clear that for numerous *in vivo* applications, a broad tropism may not be desirable. First, the tg that is encoded could be toxic to many cell types, *e.g.* pro-apoptotic or suicide genes, so a stringent control over the induction of tg expression in time and/or place is a necessity (Uch, Gerolami *et al.* 2003; Seo, Kim *et al.* 2009). A second point of concern is the risk for insertional mutagenesis; the more cells get infected, the higher this risk becomes. Although it has been demonstrated that LVs intrinsically exhibit low genotoxicity, clonal expansion and dominance of transduced hematopoietic progenitors have been reported in a clinical trial in which hematopoietic stem cells were genetically modified with a LV that expressed the β -globin gene for treatment of β -thalassemia (Fehse and Roeder 2008; Cavazzana-Calvo, Payen *et al.* 2010). Thirdly, while a broad tropism LV is favorable in anti-tumor immunotherapy to efficiently transduce antigen-presenting cells (APCs) which can induce an antigen specific immune response (Palmowski, Lopes *et al.* 2004), this is not desirable when a genetic disorder has to be restored as in this case the tg may not become an immunological target (Annoni, Battaglia *et al.* 2007). Finally, during production of pantropic viruses encoding oncogenes, narrow tropism vectors would be more valuable due to biosafety level handling requirements and safety issues (Barrilleaux and Knoepfler 2011). Therefore, in view of safety as well as applicability aspects, four main targeting strategies can be brought forward: targeted gene expression or transcriptional targeting, targeted gene translation or microRNA based (de)targeting, targeted infection or transductional targeting, and targeted integration of the proviral DNA.

2. Transcriptional targeting

Most often a strong constitutive promoter with or without enhancer sequences is used to drive the LV encoded tg. These include the cytomegalovirus (CMV), spleen focus forming virus (SFFV), human polypeptide chain elongation factor-1 α (EF-1 α), phosphoglycerate kinase (PGK) and ubiquitin C promoters (Kim, Kim et al. 2007; Gilham, Lie et al. 2010; Li, Husic et al. 2010). Although these promoters generally induce strong and ubiquitous expression of the tg, they present some disadvantages. A first drawback is that they are more prone to promoter inactivation than cell-specific promoters. In addition, they are more potent in terms of activating the host-cell defense machinery and increasing the long-distance effects of insertional mutagenesis caused by their enhancer sequences (Liu, Wang et al. 2004; Stein, Ott et al. 2010; Singhal, Deng et al. 2011). These downsides resulted in the development of various strategies to allow cell-specific tg expression by incorporating cell type specific regulatory elements and/or promoter(s) in the expression cassette of the LV. Because of the availability of a large number of endogenous cellular promoters, targeted expression can be achieved to potentially any cell type or tissue. In addition, its advantage over unselective expression has been demonstrated in numerous studies (Di Nunzio, Maruggi et al. 2008; Kerns, Ryu et al. 2010; Cao, Sodhi et al. 2011). This is exemplified by a study where LV encoding iduronidase under the control of the hepatocyte specific albumin gene promoter was injected intravenously to treat mucopolysaccharidosis type I. While the same LV with a CMV promoter resulted in the induction of an immune response that diminished the tg expression over time, the albumin gene promoter enabled stable and prolonged tg expression with a partial correction of the pathology (Di Domenico, Di Napoli et al. 2006). In addition to hepatocyte specific targeting, an ever-growing list of cell-type specific promoters has been used for the specific expression in tissues such as the erythroid lineage, endothelial cells, myocardial cells, retinal cells, B cells, epidermal, hematopoietic stem cells, *etcetera* (Hanawa, Persons et al. 2002; De Palma, Venneri et al. 2003; Semple-Rowland, Eccles et al. 2007; Di Nunzio, Maruggi et al. 2008; Leuci, Gammaitoni et al. 2009; Kerns, Ryu et al. 2010; Semple-Rowland, Coggin et al. 2010; Cao, Sodhi et al. 2011; Lee, Fan et al. 2011; Friedrich, Nopora et al. 2012).

Besides the advantage of increased and prolonged expression levels when expressed in the target cell of choice, targeted expression can also be a necessity when the tg causes undesirable damage in non-target cells. For the treatment of Mpl-deficient aplastic anemia, for example, targeted transfer to hematopoietic stem cells is inevitable since ectopic Mpl expression causes lethal adverse reactions (Heckl, Wicke et al. 2011). The same holds true for toxin, proapoptotic or suicide gene encoding LVs used in anti-tumor therapy (Zheng, Chen et al. 2003; Hsieh, Chen et al. 2011). LVs are excellent candidates to modulate the tumor and its environment since they transduce both dividing cells such as most cancer cells but also non- or very slowly dividing cells such as cancer stem cells. Furthermore LVs are able to integrate in the genome of transduced cells, potentially generating clonal populations of genetically modified cancer cells, which may then spread throughout the tumor mass (Steffens, Tebbets et al. 2004). Vector targeting can be attempted by local vector delivery, although this raises practical concerns for non-solid and metastatic tumor cells. Consequently, systemic delivery of a

targeted LVs and subsequent exclusive tg expression in cancer cells is the ultimate goal. Metastatic prostate cancer, for example, has been transcriptionally targeted in various ways (1) using a prostate-specific antigen (PSA) promoter to drive the expression of diphtheria toxin A, (2) using the prostate-stem cell antigen (PSCA) promoter to drive the expression of the Herpes Simplex Virus thymidine kinase (HSV-TK) suicide gene, or (3) combining the prostate-specific promoter ARR2PB and a short DNA sequence in the 5'-untranslated region that is recognized by the translation initiation factor eIF4E, often overexpressed in malignant cells, to drive the expression of the HSV-TK suicide gene (Yu, Chen et al. 2001; Zheng, Chen et al. 2003; Yu, Scott et al. 2006; Kimura, Koya et al. 2007; Petrigliano, Virk et al. 2009). Additionally, the tumor vasculature has been transcriptionally targeted using the endothelial specific Tie2 promoter to drive the conditionally toxic nitroreductase and subsequently diminish tumor growth (De Palma, Venneri et al. 2003). Another cancer cell type specific targeting strategy to limit tg expression to hepatocarcinoma was applied by Uch et al. They constructed a LV expressing HSV-TK under the control of the rat alpha-fetoprotein promoter elements (Uch, Gerolami et al. 2003). Besides cancer cell type specific strategies, also more generalized cancer targeting strategies have been developed. For example, as the human telomerase reverse transcriptase (hTERT) is expressed in most malignant tumors, its promoter has been used to drive the expression of the cytosine deaminase gene together with a green fluorescent protein (GFP) reporter gene. It was demonstrated that hTERT-positive tumors could be visualized after intratumoral injection of the LV in tumor-bearing nude mice and, more importantly, that significant tumor growth suppression was observed after delivery of the pro-drug 5-fluorocytosine (Yu, Li et al. 2011). Besides avoidance of toxic tg expression in a non-tumor cell, tumor specific gene therapy is also interesting for targeted imaging. For example, the use of the chimeric promoter EIIAPA containing the alpha-fetoprotein promoter and hepatitis B virus enhancer II was used to control the downstream expression of luciferase genes to subsequently assay the selective transcriptional activity by bioluminescence imaging (Hsieh, Chen et al. 2011).

As LVs efficiently infect non-dividing cells, they provide suitable platforms for tg delivery to multiple mammalian neuronal cell types. It has been shown that stereotactic injection of LVs in the brain parenchyma leads to transduction of the striatum, hippocampus and thalamus (Watson, Kobinger et al. 2002). Moreover, transcriptional targeting has proven to be a reliable technique to unravel the complexity of the nervous system by neuron and brain specific assessment of the effects of therapeutic proteins and RNA interference, or to investigate neuronal gene expression (Hioki, Kameda et al. 2007; Gascon, Paez-Gomez et al. 2008; Kuroda, Kutner et al. 2008; Peviani, Kurosaki et al. 2012). Regulatory sequences of rat neuron specific enolase, human glial fibrillary acidic protein and myelin basic protein have already been exploited to obtain LV-mediated selective gene targeting of neurons, astrocytes and oligodendrocytes, respectively (Jakobsson, Ericson et al. 2003; McIver, Lee et al. 2005). This has led to applications like subregional tg expression in the hippocampus using the hybrid hEF1alpha/HTLV promoter or neuron specific synapsin I promoter or targeting the central serotonergic neurons using a two-step transcriptional amplification strategy co-expressing the tryptophan hydroxylase-2 gene promoter with the chimeric enhancer GAL4/p65 (Kuroda, Kutner et al. 2008; Benzekhrroufa, Liu et al. 2009). Next to the central nervous system, Bend-

otti et al. recently focused on selective tg expression in mouse spinal cord motor neurons using motor neuron specific regulatory sequences derived from the promoter of the homeobox gene Hb9 (Benzekhoufa, Liu et al. 2009; Peviani, Kurosaki et al. 2012). However, neuron specific gene expression is not always very efficient and therefore several groups have attempted to improve the endogenous promoters using extra enhancers or artificial transcriptional activators such as the bidirectional promoter. For the latter, Liu et al. based their bidirectional promoter on the transcriptional activity of the human synapsin-1 promoter and the compact glial fibrillary acidic protein (GfaABC1D) promoter. In the opposite orientation, a minimal core promoter of 65 basepairs (bp) derived from the CMV promoter was joined upstream of both promoters, which were flanked with two gene expression cassettes. The 5' cassette transcribed the artificial transcriptional activator while the downstream cassette drove the expression of the gene of interest (Liu, Paton et al. 2008).

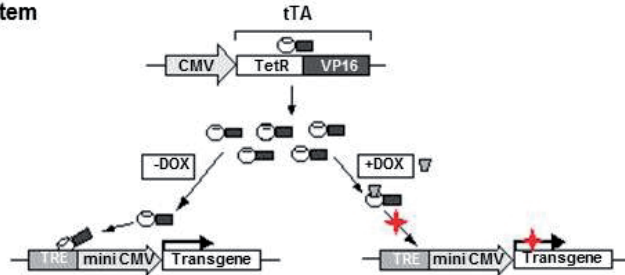
To fulfill the high expectations of gene therapy, both efficient delivery and sustained expression of the therapeutic gene are essential requirements. However, one of the major barriers to stable gene transfer by LVs is the development of innate and adaptive immune responses to the delivery vector and the transferred therapeutic tg. It became clear that *in vivo* administered broad tropism LVs efficiently transduce APCs and that these play a major role in the induction of tg specific immune responses (Annoni, Battaglia et al. 2007; Vandendriessche, Thorrez et al. 2007). Consequently transcriptional targeting can be applied to avoid tg expression in APCs. Brown et al. demonstrated stable GFP production by modified cells *in vivo* when tg expression was prevented in APCs (Brown, Venneri et al. 2006). Another study combined the hepatocyte specific enhanced transthyretin promoter with an APC-detargeting microRNA strategy, and showed the induction of GFP-specific regulatory T cells and the promotion of immunological tolerance (Annoni, Brown et al. 2009). Moreover, Matrai et al. demonstrated that hepatocyte-targeted expression by an integrase-defective LV (IDLV) induced tolerance to coagulation factor IX with prevention of the induction of neutralizing antibodies in mice (Matrai, Cantore et al. 2011). In contrast to gene therapy, immunotherapy pursues the induction of a tg-specific immune response where APC-specific transduction is imperative. Therefore, LVs that drive tg expression *via* an APC-specific promoter have been developed. For instance Cui et al. used the HLA-DR promoter to target human MHC class II⁺ cells like dendritic cells (DCs, CD83⁺) and macrophages (CD14⁺). They demonstrated the induction of an allogeneic T cell response *in vitro* (Cui, Golob et al. 2002). The dectin-2 promoter was used to target the expression of the human melanoma antigen NY-ESO-1 to murine APCs. After intravenous injection of the targeted LVs, selective tg expression in dectin-2⁺ splenic myeloid and plasmacytoid DCs as well as in F4/80⁺ macrophages was reported. Furthermore CD11c⁺ draining lymph node residing DCs were targeted after subcutaneous injection which resulted in strong NY-ESO-1 specific CD8⁺ and CD4⁺ T cell responses (Lopes, Dewannieux et al. 2008). On the other hand, DC-induced tg specific tolerance has also been achieved after the use of a DC-specific promoter. When LVs carrying a CD11c promoter were used to make DC-specific transgenic mice by injecting the purified virus into the perivitelline space of single-cell embryos, the tg became an autologous antigen to

which immunological tolerance was induced. Furthermore, this tg was only expressed in CD11c⁺ cells derived from the spleen, lymph nodes as well as the thymus (Zhang, Zou et al. 2009). Dresch et al. made use of the DC-STAMP promoter to engineer bone marrow-targeted LVs. Therefore, *ex vivo* transduced hematopoietic stem cells (HSC) were injected in lethally irradiated mice to make HSC chimeric animals (Dresch, Edelmann et al. 2008). When GFP expression was analyzed in the leukocyte population isolated from the spleen, the main DC subpopulations such as CD11b⁺CD8⁺ DCs, CD11b⁺CD8⁻ DCs, and plasmacytoid DCs were GFP positive next to a small percentage of CD11c⁺CD11b⁺ monocytes. Furthermore, tg expression could only be detected in CD11c⁺ cells in the thymus. While the previous two tolerance inducing studies could be explained by the fact that undifferentiated DC precursors were transduced, Kimura et al. intravenously injected LVs encoding Trp2 driven by the MHCII promoter and also observed persistent tg expression selectively in the CD11c, CD11b and CD19⁺ MHCII⁺ cells of the spleen without CD8⁺ T cell responses against Trp2 in contrast to a CMV carrying construct (Kimura, Koya et al. 2007; Dresch, Edelmann et al. 2008). The induction of tolerance in this study might be explained by the activation status of the transduced APCs. Induction of tg specific effector T cells requires fully activated APCs. Since, DC activation by LVs was shown to be dose-dependent, the LV titers used in these studies could explain the tolerogenic instead of stimulatory outcome (Breckpot, Emeagi et al. 2007; Breckpot, Escors et al. 2010).

Finally, also controllable or inducible tg expression can be a prerequisite. Reasons to use tg regulation are: to maintain appropriate levels of a gene product within the therapeutic range, to modulate, stop or resume tg expression in response to disease evolution, or in response to an endogenous molecule as *e.g.* the secretion of insulin induced by hyperglycemia. For human gene therapy, several ligand dependent transcription regulatory systems have been developed. For clinical applications, such systems need to be safe, specific, highly inducible, reversible and only show dose dependent activation with low basal activity while their ligands need to be bioavailable and low in immunogenicity (Toniatti, Bujard et al. 2004). One of the first and most widely used ligands is Tetracyclin (Tet) or its more potent analog Doxycycline (Dox) (Efrat, Fusco-DeMane et al. 1995; Reiser, Lai et al. 2000). In contrast to the bacterial lac repressor/operator or the Cre-loxP recombinase system, it is applicable *in vivo* and reversible (Deuschle, Hippskind et al. 1990; Lakso, Sauer et al. 1992). The original bacterial Tet system is based on a Tet repressor protein (TetR) that inhibits the expression of the bacterial Tet resistance genes by binding to cognate operator sequences (TetO) in their regulatory regions. Upon the addition of Tet, the repressor is inactivated by allosteric change, allowing gene transcription (Gossen and Bujard 1992). The artificial Tet-off system is based on the generation of a hybrid transactivator (tTA) by fusion of the TetR to the transcription activation domain of the HSV VP16 protein. This fusion product will bind and activate transcription at promoters that include TetO while the presence of Dox impairs this binding, resulting in the shut off of gene expression (Furth, St Onge et al. 1994) (Figure 2A, adapted from (Ramezani and Hawley 2002). In contrast, the reverse Tet transactivator (rtTA), generated by ran-

dom mutagenesis of tTA, requires Dox to bind to cognate operator sequences and activate transcription resulting in the inducible Tet-on system (Figure 2B).

A) Tet-off system



B) Tet-on system

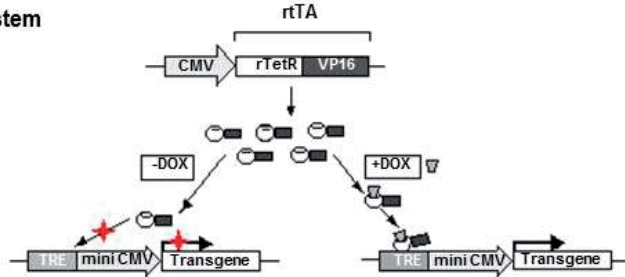


Figure 2. Representation of the artificial Tet-off (A) and Tet-on system (B). While the Dox binding transactivator (tTA) binds to the tetracycline-responsive promoter element (TRE) and stimulates tg transcription in the absence of Dox(A), the mutant reverse Tet-controlled transactivator (rtTA) binds to the TRE in the presence of Dox and stimulates transcription(B).

However, the *in vivo* applicability of the Tet system remained limited due to leakiness and insufficient induction levels. Therefore the Tet-on system has been optimized *e.g.* by isolating novel rtTA variants and incorporating a Dox-dependent trans-silencer called tTS which consists of the KRAB (Krüppel-Associated Box) trans-repressing domain of the human Kid-1 protein fused to the wild type TetR. This tTS has been used by the group of Szulc et al. to develop a LV-based conditional gene expression system for drug-controllable expression of inhibitory short hairpin RNAs (shRNAs), and reported on a robust and versatile system that governed the tight control over the tg expression both *in vitro* as well as *in vivo* among others to generate transgenic mice (Szulc, Wiznerowicz et al. 2006). Moreover, Dox is orally bioavailable, has a half-life of 14-22 hrs and has an excellent tissue penetration. Therefore numerous groups have used both the Tet-on and Tet-off system within LV-based gene reporter and therapeutic applications (Blomer, Naldini et al. 1997; Bahi, Boyer et al. 2004; Blesch, Conner et al. 2005; Liu, Wang et al. 2008; Hioki, Kuramoto et al. 2009; Adriani, Boyer et al. 2010). This is exemplified by a study of Seo et al. who developed an oncolytic LV-

mediated Tet-on inducible system based on co-transduction of two LVs to drive the expression of a pro-apoptotic gene by the promoter of matrix-metalloproteinase-2 (MMP-2), which is highly expressed in several cancer cell lines. The first LV expressed a rtTA under the control of the MMP-2 promoter, whereas the second LV expressed the pro-apoptotic gene Bax, under the control of the tetracycline-responsive element (Seo, Kim et al. 2009). While most Dox inducible systems are based on the co-transduction of two LVs, all-in-one vectors have also been described recently (Ogueta, Yao et al. 2001; Barde, Zanta-Boussif et al. 2006; Herold, van den Brandt et al. 2008; Wiederschain, Wee et al. 2009; Benabdellah, Cobo et al. 2011). Furthermore, an extra Dox-regulated system based on the original TetR protein was developed in 1998. It serves as an alternative to the tTA- and rtTA-based systems because the latter were accompanied by secondary effects due to expression of the transactivator domains. Benabdellah et al. made use of the Dox-responsive cassette driving the expression of eGFP and the SFFV promoter expressing high amounts of the TetR protein in an all-in one vector system. This LV efficiently produced Dox-regulated cell lines, including primary human fibroblasts and human mesenchymal stem cells. However, a major concern using Dox remains the possibility to develop resistance to the antibioticum Tet, and although it seems a non-immunogenic system in several mouse strains, studies with intramuscularly delivered Tet-on activators in non-human primates did elicit a cellular and humoral response (Latta-Mahieu, Rolland et al. 2002).

Besides the Tet on/off systems, a plethora of inducible systems has been examined both *in vitro* and *in vivo*. An interesting strategy is based on the use of small molecules with distinct binding surfaces for two different polypeptides to modulate the activity of dimerizer-regulated systems. The prototype molecule is rapamycin, which mediates the heterodimer formation between two molecules (FK506-binding protein and FKBP rapamycin binding) that are coupled to a DNA binding domain (DBD) and transcription activation domain (AD) respectively (Pollock, Issner et al. 2000). The rapamycin inducible system has low basal activity because of the physical separation of the DBD and AD molecules, the ligand has a short half-life of about 4.5 hrs although the induced gene expression lasts for days due to the strong stability of the DBD-AD assembled complex (Toniatti, Bujard et al. 2004). Tian et al. used a variant of this system to engineer LVs that produce a fusion protein between the furin-cleavable proinsulin and the self-dimerization mutant of FK506-binding protein to yield bioactive insulin in keratinocytes. Epidermal keratinocytes in culture, in stratified bioengineered epidermis as well as implanted in diabetic athymic mice released insulin within maximally 1 hr after addition of rapamycin. Secretion slowed or stopped within 2-3 hrs after removal of the inducing agent. Even in diabetic animals with severe hyperglycemia, decreased serum glucose levels to normal levels were reported (Tian, Lei et al. 2008). The major disadvantage of this technique is the immunosuppressing activity of rapamycin and the only partial oral availability, which renders this system impractical for clinical applications.

Another strategy is based on the fact that heterologous proteins can be made hormone responsive by fusing them with the hormone-binding domain of steroid receptors. The best-characterized system is regulated by mifepristone or RU486, a synthetic progesterone antagonist. Prototypically the RU486-binding chimera known as GeneSwitch® consists of the

GAL4 DBD from *Saccharomyces cerevisiae* fused to the ligand-binding domain of a mutant progesterone receptor and the activation domain of the p65 subunit of human NF- κ B (Abruzzese, Godin et al. 2000; Sirin and Park 2003). Upon ligand binding the GeneSwitch® protein binds to GAL4 upstream activating sequences in the promoter driving the expression of the tg of interest. An advantage of the GeneSwitch® system is that the majority of its components are modified human proteins with no impact on cell viability. Furthermore, usage of a mifepristone-inducible (auto-inducible) promoter to regulate expression of the chimeric transactivator dramatically reduced basal expression of the tg in the absence of the inducer, thereby improving the dynamic range of *in vivo* tg regulation (Shinoda, Hieda et al. 2009). In addition, although mifepristone has anti-progesterone and -glucocorticoid activities, the concentration needed for ligand-inducible transactivation of the target gene is much lower than the concentration producing an anti-progesterone effect in humans. However, it is thought that the lower dosage may still affect the ovarian cycle and exert a contraceptive activity. Therefore the search for other inducers that are unable to interact with endogenous progesterone would be more appropriate for clinical use (Sarkar 2002). As an alternative steroid-receptor based inducible system, the glucocorticosteroid responsive element (GRE5) was cloned into a LV (LV-GRE-IL10) encoding interleukin-10 (IL-10). Expression of IL-10 by LV-GRE-IL-10 appeared rapidly, was sustained and inducible in both ovine and human corneas in the presence of dexamethasone (Parker, Brereton et al. 2009). Another alternative can be the steroid hormone ecdysone, which plays a fundamental role during insect molting and metamorphosis. Ecdysteroids are considered safe because they are found in large amounts as phytoecdysteroids in vegetables, present in the human diet without detrimental effects. Mouse hematopoietic progenitors transduced with LVs containing an ecdysone-regulated GFP expression cassette efficiently turned GFP expression on and off in transplanted animals with low basal activity (Xu, Mizuguchi et al. 2003; Galimi, Saez et al. 2005). Possibly, several other systems will be developed to control tg expression after LV transduction. Potential systems could be based on the cell-cell communication quorum sensing process (Neddermann, Gargioli et al. 2003) or the naturally evolved mechanisms of antibiotic resistance to pristinamycin, a composite streptogramin antibiotic or erythromycin, a member of the macrolide antibiotics (Fussenegger, Morris et al. 2000; Roberts 2002).

3. microRNA detargeting

Recently, the concept of microRNA (miRNA) mediated post-transcriptional tg regulation was introduced in LV-based targeting. miRNAs are 21-22 nucleotide long non-coding fragments which are partially or extensively complementary to an endogenous mRNA molecule (Lai 2002). In mammals, over 400 different miRNAs have been identified so far, most of which are well conserved among species ranging from plants, worms, insects to humans (Brown and Naldini 2009). Some of these miRNAs are expressed ubiquitously whereas others are only expressed at certain developmental stages or in a certain cell type. Upon binding of a miRNA molecule to its complementary target sequence, repression of translation or direct destruction of the mRNA is induced. The detailed mechanisms involved in this post-

transcriptional regulation process, do not lie within the scope of this book chapter but are reviewed elsewhere (Nelson, Kiriakidou et al. 2003; Bartel 2004). A brief description together with a schematic representation is depicted in Figure 3 (adapted from <http://www.micro-rna.ic.cz/mirna4.html>).

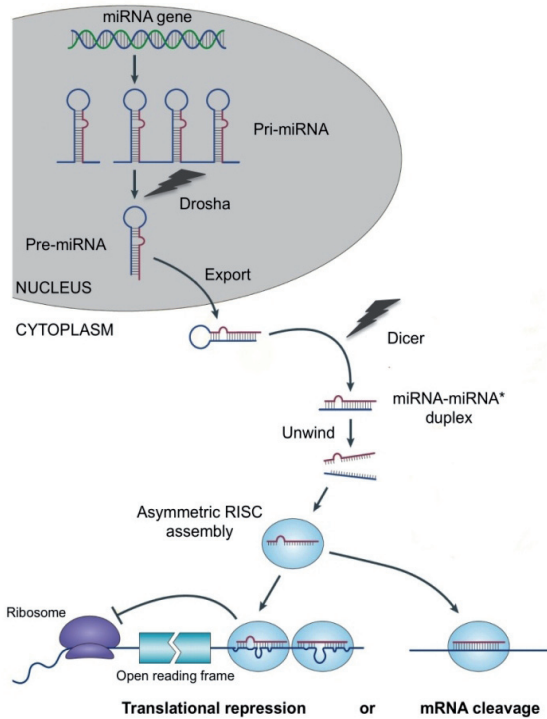


Figure 3. miRNA-based post-transcriptional gene silencing. Briefly, endogenous miRNA genes are transcribed by RNA polymerase II to pri-miRNA precursor molecules in the nucleus. These are processed to pre-miRNA by a specialized enzymatic pathway called Pasha/Drosha and will release the pre-miRNA in short hairpin RNA (shRNA). Then, these pre-miRNAs are exported to the cytoplasm where Dicer degrades most of the shRNA, leaving a miRNA duplex which is loaded onto the AGO complex (Argonaut), forming the preRISC (RNA Interference Silencing Complex). Subsequently the miRNA strand is degraded, leaving its complementary miRNA intact within the RISC complex. Then, this complex scans mRNAs and when complementation is found, the mRNA is degraded or the poly-A tail is removed, leading to mRNA destabilization or stalled mRNA translation.

In order to limit undesired vector tg expression, LVs encoding target sequences of endogenous miRNAs have been developed. By incorporating at the 3' UTR region of the expression cassette one or more copies of a sequence that is perfectly complementary to a miRNA (miRNA tagging), the transgenic mRNA will be degraded or repressed in cells where the complementary miRNA is expressed. This new way of controlling tg expression at the level of the

mRNA product came as a complementary strategy to transcriptional targeting since the latter is associated with some disadvantages such as: (1) difficulty to identify and faithfully reconstitute a gene's promoter; (2) for integrating LVs, promoters and enhancers can be trapped, leading to aberrant expression (De Palma, Montini et al. 2005), (3) transcription can be promiscuous and (4) only few genes have truly cell-specific transcriptional patterns while several promoters are active in many different cell types or states. Moreover, as miRNAs regulate expression at the post-transcriptional level, copy number and vector integration site have no appreciable effect on their regulation, which ensures consistent control throughout the transduced cell population.

Successful outcomes of LV-based gene therapy have long been precluded by the development of tg-specific immunity as a consequence of the direct expression of the tg product by professional APCs. Therefore Brown et al. challenged mice with LVs encoding a target sequence for miRNA-142-3p, a microRNA specifically expressed in the hematopoietic lineages. Upon injection, they demonstrated a 100-fold suppression of reporter gene expression in intravascular and extravascular hematopoietic lineages, including APCs (Brown, Venneri et al. 2006). One year later, its usefulness was evidenced by the miRNA-142-3p regulated LV mediated stable correction of hemophilia B in mice (Brown, Cantore et al. 2007). Its expression leads to reduced tg expression in APCs and subsequently lower anti-tg immune responses. Moreover it was demonstrated that *in vivo* delivery of this post-transcriptionally regulated LV induced tg-specific Foxp3⁺ regulatory CD4⁺ T cells, which promoted immunologic tolerance (Annoni, Brown et al. 2009). Curiously, they also reported the necessity of a hepatocyte specific promoter for this immunological tolerance. So, these studies showed the impressive potential of miRNA-based detargeting to overcome a major hurdle for clinical gene therapy, however also other factors than tg expression in APCs seem to influence the immunological outcome of a gene transfer procedure. Examples are the type of vector used, the tissue targeted and the presence of inflammation (Brown and Lillicrap 2002; Cao, Furlan-Freguia et al. 2007).

Another reason to pursue stringent tg regulation, is to express the tg in a specific developmental state. Brown et al. showed that multiple endogenous miRNAs can be used to achieve tg expression patterns that rapidly adjust and sharply discriminate among the myeloid and lymphoid lineage in therapeutically relevant HSCs and their progeny with miRNA-223, or among immature and mature APCs using miRNA-155 (Brown, Gentner et al. 2007). Another example is provided by Gentner et al. who used the miRNA-126 target sequence to detarget tg expression from stem cells and progenitors from the hematopoietic cell lineage in order to avoid expression of the highly toxic GALC in these stages, while inducing GALC expression in mature cells from the hematopoietic lineage to correct globoid cell leukodystrophy (Gentner, Visigalli et al. 2010). Furthermore the group of Sachdeva et al. used miRNA-292 regulated LVs to visualize and segregate differentiating neural progenitors in pluripotent cultures and demonstrated that miRNA-regulated vectors allow a potentially broad use on stem cell applications (Sachdeva, Jonsson et al. 2010). Finally, Sadelain et al. used LVs that encode antigen specific receptors together with target sites for miRNA-181a to suppress the expression of the receptor in late thymocytes. This avoided clonal deletion of antigen specific T cells in

the thymus and subsequent challenge with antigen expressing tumors did not result in tumor growth (Papapetrou, Kovalovsky et al. 2009).

Furthermore this technology is useful as a mechanism to increase vector safety and efficacy by limiting the expression of a toxic or pro-apoptotic tg to certain target cells. For example Lachmann et al. used the miRNA-150 target sequence to suppress GFP expression in lymphocytes and thereby prevented tg-induced lymphotoxicity (Lachmann, Jagielska et al. 2011). On the other hand unrestrained growth of transduced cells could also be avoided using miRNA-based detargeting when growth-promoting gens are replaced (Hawley, Fong et al. 1998). Moreover, miRNA-based regulation could be desirable when targeted gene expression is needed to assess the contribution of a particular cell type to physiological processes or for the development of new therapeutic strategies. This is exemplified by the work of Colin et al. who segregated tg expression between neurons and astrocytes following injection into the brain by exploiting the activity of miRNA-124 (Colin, Faideau et al. 2009). Another miRNA-based targeting strategy developed a few years ago was the concept of miRNA sponges, decoys, erasers, antagomirs or knockdowns (Ebert, Neilson et al. 2007; Scherr, Venturini et al. 2007; Gentner, Schira et al. 2009). Therefore vectors expressing miRNA target sites can effectively saturate an endogenous miRNA and prevent it from regulating its natural targets. This technology enables a new way of investigating miRNA biology and has already been used to study the role of miRNAs in cancer, cardiac function and hematopoiesis (Scherr, Venturini et al. 2007; Bonci, Coppola et al. 2008; Kumar, Erkeland et al. 2008; Sayed, Rane et al. 2008; Gentner, Schira et al. 2009; Valastyan, Reinhardt et al. 2009).

A possible concern of miRNA-based detargeting is whether sufficient target knockdown can be achieved for specific applications without escape mutants arising (Kelly, Hadac et al. 2008). In addition, it is highly likely that overexpression of the synthetic target sites will saturate their corresponding endogenous miRNAs and deregulate expression of natural targets with deleterious consequences. However, the latter has not been reported so far (Brown, Gentner et al. 2007). Moreover, miRNA-based regulation is a very robust system since at low copy vector number miRNA regulation of tg expression remains effective. Apparently, when a threshold miRNA concentration is present, the tg will be suppressed. This robustness can probably be explained by the perfect complementarity of the target sequence and the endogenous miRNA sequence. Indeed, when imperfectly complementary sites were used, this did result in a detectable decrease in target suppression, although only at very high vector copy numbers. So, although it should be recognized that the knowledge regarding miRNA biology and function is still limited, this strategy holds great potential to carefully move towards clinical translation (Brown and Naldini 2009)

4. Transductional targeting

Although the strategies described above demonstrate cell-specific gene expression, they often require broad tropism LVs which does not reduce the risk for RCL formation or insertional mutagenesis. Therefore transductional targeting of LVs seems a more interesting

strategy to tackle both safety and efficacy concerns. The concept of swapping the viral envelope proteins of different viral species is called pseudotyping. Already in 1979, the envelope glycoprotein of the avian retrovirus was used to pseudotype VSV virions in order to selectively enrich for VSV temperature-sensitive mutants of VSV.G biosynthesis (Lodish and Weiss 1979). Later it was shown that wild type HIV-1 particles which were produced in cells that were infected with another virus, *e.g.* murine leukemia virus (MLV) or VSV, led to the generation of phenotypically mixed virions with an expanded host range (Canivet, Hoffman et al. 1990; Zhu, Chen et al. 1990). These observations introduced the concept of pseudotyping and in the early 90's the gp160 sequence of a replication defective HIV-1 derived LV was replaced by a MLV gp (Page, Landau et al. 1990). Later on the natural envelope gp from an MLV-based vector was replaced with the viral attachment protein of VSV (Emi, Friedmann et al. 1991; Burns, Friedmann et al. 1993). Today, most synthetic LVs are pseudotyped with a heterologous envelope protein to increase their stability, infectivity and safety. Notably, the first LVs were not pseudotyped but displayed the native HIV-1 envelope protein at their surface. This limited their tropism to CD4-expressing cells (Dropulic 2011). Interestingly, VSV.G pseudotyped vectors are more stable than their natural counterparts. This allows concentration to higher titers by ultracentrifugation and confers broad tropism, as VSV.G binds to a still unknown ubiquitous membrane component (Cronin, Zhang et al. 2005). This superior transduction efficiency comes in handy for the treatment of genetic disorders such as β -thalassemia and X-linked adrenoleukodystrophy (Cartier, Hacein-Bey-Abina et al. 2009; Cavazzana-Calvo, Payen et al. 2010). Nonetheless, VSV.G pseudotyped LVs also present several downsides. Firstly, the VSV gp is cytotoxic when expressed constitutively at high concentrations, which impedes the production of stable packaging cell lines (Lopes, Dewan-nieux et al. 2011). In addition, cytotoxicity associated with VSV.G pseudotyped LVs has been observed when *in vivo* administered at high concentration, in comparison with other pseudotypes (Watson, Kobinger et al. 2002). Another critical hurdle for systemic delivery using VSV.G pseudotyped LVs is their susceptibility to neutralization by human serum complement, although this can be bypassed by polyethylene glycol-modification (PEGylation) of the virions (DePolo, Reed et al. 2000; Croyle, Callahan et al. 2004).

An ever-growing list of alternative pantropic as well as ecotropic naturally occurring gps has been evaluated for LV pseudotyping. These vary in origin, tropism, titer, stability, efficiency of packaging, inactivation by complement, efficiency of cell transduction and induction of an immune response (Cronin, Zhang et al. 2005). They can be of retroviral origin such as those from T-lymphotropic virus, maedi-visna virus, MLV, feline endogenous retrovirus and gibbon ape leukemia virus (GALV) (Rasko, Battini et al. 1999; Stitz, Buchholz et al. 2000; Zeilfelder and Bosch 2001; Strang, Ikeda et al. 2004; Sakuma, De Ravin et al. 2010). In general, LVs pseudotyped with a γ -retroviral envelope transduce CD34⁺ hematopoietic precursors, a property that has been used for the correction of X-linked severe combined immunodeficiency (SCID) using the GALV or MLV-A envelopes (Cavazzana-Calvo, Hacein-Bey et al. 2000; Gaspar, Parsley et al. 2004). Nonetheless, envelope gps of numerous non-retroviral families have been used as well to pseudotype LVs. A first example is provided by the *Togaviridae* family, where their envelope gps (from alphaviruses such as the Ross River virus) equips the LV with a mouse and human DC-specific tropism when injected intravenously

(Strang, Takeuchi et al. 2005), and with an astrocyte and oligodendrocyte specific tropism when injected into the mouse brain (Kang, Stein et al. 2002). Another example is provided by the family of the *Baculoviridae* where the gp64 gp ensures high particle stability in addition to a hepatocyte specific tropism (Matsui, Hegadorn et al. 2011). LVs pseudotyped with the lymphocytic choriomeningitis virus (LCMV) envelope from the *Arenaviridae* preferentially transduce cells from the central nervous system such as neural stem cells and progenitor cells, and also to glioma cells and insulin secreting β -cells (Kobinger, Deng et al. 2004; Miletic, Fischer et al. 2004; Stein, Martins et al. 2005). As there is an increasing interest in the development of gene therapeutic strategies for malignant gliomas, the most frequent primary brain tumors with very poor prognosis, several groups report on the use of LCMV gp pseudotyped LVs to target almost exclusively astrocytes, the main source of malignant glioma cells (Beyer, Westphal et al. 2002; Miletic, Fischer et al. 2004; Steffens, Tebbets et al. 2004). The H and F envelope proteins from the *Paramyxoviridae* family, such as those derived from measles viruses, provide LVs with the capacity to bind to SLAMF and CD46, which confers efficient virus entry, nuclear transport and integration in non-activated B and T lymphocytes. This property is particularly important, since primary unstimulated B and T cells are generally difficult to transduce if not pre-treated to induce progression through the cell cycle (e.g. through stimulation with anti-CD3/anti-CD28 antibodies or cytokines) (Frecha, Levy et al. 2010; Frecha, Levy et al. 2011). To transduce airway epithelial cells efficiently, envelope proteins from several viruses that infect respiratory tissues or cells have been evaluated. For efficient transduction of unconditioned airway epithelial cells from the apical side, envelopes derived from the ebola virus (*Filoviridae*), members of the *Paramyxoviridae* such as the respiratory syncytial (RSV) and sendai viruses, and members of the *Orthomyxoviridae* such as the influenza and fowl plaque viruses have been evaluated (Kobinger, Weiner et al. 2001; Neffkens, Garcia et al. 2007; Mitomo, Griesenbach et al. 2010). Surprisingly, it has been reported that the S protein of the severe acute respiratory syndrome-associated coronavirus (*Coronaviridae*) mediates entry into hepatoma cell lines (Hofmann, Hattermann et al. 2004). Finally, although the vesicular stomatitis, morbilli and rabies virus are all derived from the *Rhabdoviridae* family, only LVs pseudotyped with the rabies-G envelope enable retrograde transport to motoneurons of the spinal cord upon intramuscular injection or to the thalamus upon striatal injection. In contrast, VSV.G displaying LVs transduce cells only locally while morbilli-pseudotyped LVs preferentially target non-neuronal glial cells (Mazarakis, Azzouz et al. 2001; Azzouz, Ralph et al. 2004; Wong, Azzouz et al. 2004; Colin, Faideau et al. 2009; Calame, Cachafeiro et al. 2011).

Although the use of an existing viral envelope gp seems the most straightforward way to pseudotype LVs, a natural variant with the desired delivery properties is not available for every therapeutic application. Moreover, natural gps can come with limitations such as sensitivity to neutralization by the host immune response, lack of specificity and/or insufficient transduction efficiency. Also their production and purification can be inefficient (Schaffer, Koerber et al. 2008). Therefore, the development of LVs with customized, user-defined gene delivery properties by molecular engineering of the envelope gps is an alternative strategy to retarget the LV to specific cell-surface receptors. This molecular engineering has become a collective term for many different strategies, which will be described below.

A first strategy to alter the tropism of a virally derived gp is by rational point and domain mutagenesis. This is exemplified by the DC-specific targeting strategy from Yang et al. Certain subsets of DCs carry the DC-SIGN protein (also known as CD209) on their surface, which is a C-type lectin-like receptor that potentiates rapid binding and endocytosis of materials. The sindbis virus envelope gp consists of two integral membrane gps that form a heterodimer and function as one unit. The fusogenic monomer is E1 and needs binding *via* E2 to mediate low pH-dependent fusion. The latter binds to the DC-SIGN receptor, next to the canonical viral receptor heparin sulfate, expressed by many cell types. Since both protein binding sites are physically separated, selective mutation at the E2 monomer is possible, abrogating the heparin sulfate binding part while leaving the DC-SIGN binding part intact. By pseudotyping a LV with this mutated sindbis virus derived envelope gp, targeted infection of DCs *in vivo* after direct subcutaneous administration was achieved. Moreover, this elicited a strong antigen-specific immune response (Yang, Yang et al. 2008; Hu, Dai et al. 2010). Another example is the substitution of the V3-loop region of the SIV envelope gp with the corresponding region of a T cell tropic HIV-1 to create a T-cell targeted MLV vector, pseudotyped with this engineered SIV gp (Steidl, Stitz et al. 2002). A final example is provided by Dylla et al. who diminished the α -dystroglycan affinity of the LCMV WE45 strain envelope gp by a point mutation. When a FIV derived LV was pseudotyped with this point mutated LCMV gp, their intravenous injection in adult mice yielded low transduction efficiencies in hepatocytes in contrast to abundant liver and cardiomyocyte transduction with the wild type LCMV gp pseudotyped FIVs (Dylla, Xie et al. 2011).

Apart from genetic alterations, chemical modifications can also alter LV tropism. PEGylation of VSV.G pseudotyped LVs is one such example where the LVs' tropism is not altered. Nevertheless, chemical modifications can lead to targeted gene delivery vehicles, for example by tagging the MLV vector with galactose to selectively transduce human hepatoma cell lines expressing asialo-gp receptors specific for oligosaccharides with terminal galactose residues (Neda, Wu et al. 1991). Furthermore, Morizono et al. reported the production of LVs pseudotyped with sindbis virus gps in the presence of deoxymannojirimycin. This modification altered the structures of N-glycans from complex to high mannose structures as it inhibits mannosidase. This led to DC-SIGN specific binding although the gps were genetically modified to prevent interaction with DC-SIGN (Morizono, Ku et al. 2010). Furthermore it was demonstrated that binding of sindbis virus gp to DCs is directly related to the amount of high-mannose structures on the gp (Tai, Froelich et al. 2011). Unfortunately, the effectiveness of the chemically modified particles strongly depends on the reaction conditions of the applied modifications.

Other chimeric envelope gps can be generated by covalently fusing a short peptide, a ligand or an antibody to an envelope gp. The advantages of short peptides are that they don't severely disrupt the original envelope gps' function and that *via* high-throughput library approaches, targeted peptides with strong binding affinity and unlimited specificity within the context of a particular gp can be generated (Schaffer, Koerber et al. 2008). However, they can hinder multimerisation of capsid monomers, create fusion products with lower thermostability and hinder proper intracellular trafficking of the gp during viral production. The latter

is exemplified by the blockage in trafficking in the producer cells to the plasma membrane of VSV.G when linked to a collagen-binding motif (Guibinga, Hall et al. 2004). Different kinds of ligands such as cytokines and growth factors have been linked to the amino-terminal region or receptor-binding domain of the envelope gp, most often derived of MLV. This is amongst others exemplified by fusion of the MLV gp to hepatocyte growth factor to target the LV to hepatocytes (Nguyen, Pages et al. 1998), or to the insulin-like growth factor (IGF-I) (Chadwick, Morling et al. 1999). Interestingly, these ligands can elevate the transduction efficiency by altering the targets' physiological state. When the fusion product of the MLV gp and IL-2 is used to pseudotype LVs, a 34-fold higher infection efficiency was observed of quiescent IL-2 receptor expressing cells compared to LVs pseudotyped with the wild type MLV gp. This was explained by IL-2 induced activation of the cell cycle from the otherwise barely transducible quiescent cells (Maurice, Mazur et al. 1999). However, a very low to unobservable transduction profile is often reported which can be attributed to sequestration of the LV particles at the target cell surface, directing the viral particle to a degradation pathway after endocytosis and/or inability of the fusion product to trigger a conformational change essential for viral fusion and subsequent infection (Lavillette, Russell et al. 2001; Katane, Takao et al. 2002). In addition to peptides and ligands, also antibodies and their derivatives can be used. In general, single chain variable fragments or scFvs offer higher specificity than short peptides but as they are larger in size, the chance that they disrupt the process of conformational changes of the gp to mediate membrane fusion increases. Therefore scFvs are most often linked to a spacer peptide that permits proper conformation of both the scFv domain and the envelope gp as exemplified by the fusion of the MLV gp to a scFv against MHC class I (Karavanas, Marin et al. 2002). For LV targeting to APCs, several attempts have been made to couple an anti-MHC class II scFv to an ecotropic gp such as MLV or VSV.G (Dreja and Piechaczyk 2006; Gennari, Lopes et al. 2009). Recently, the use of DARPins or designed ankyrin repeat proteins has been reported. These can be fused to the H protein of measles virus for example and then be co-displayed with the fusogenic F protein on the surface of the LV. The advantage is that DARPins can be selected to become high-affinity binders to any kind of target molecule thus this seems a promising alternative to scFvs for retargeting LVs (Munch, Muhlebach et al. 2011). So, in general, the use of chimeric envelope proteins for LV targeting has proven to offer tremendous opportunities but at the same time to be a challenge as the function of chimeric gps is often severely compromised which leads to a very inefficient transduction profile (Fielding, Maurice et al. 1998; Dreja and Piechaczyk 2006; Waehler, Russell et al. 2007; Buchholz, Duerner et al. 2008).

Several solutions have been created to circumvent the problems associated with the formation of conformational dysfunctional fusion products. One solution is the inclusion of a protease cleavable peptide between the gp and the ligand. This is certainly an interesting strategy for the targeting of tumor cells, as they secrete MMP, which degrade the extracellular matrix to metastasize. By linking a proline-rich hinge and an MMP cleavage site to the fusion product of a scFv recognizing carcinoembryonic antigen (CEA) and the MLV gp, selective targeting of CEA-positive cells after *in vivo* injection of producer cells at the tumor site was observed (Chowdhury, Chester et al. 2004). Taking this hinge region idea one step further, the concept of 'molecular bridges' was introduced where a bispecific linker mole-

cule recognizes both the viral gp as well as the molecular determinant on the target cell. This concept is based on a bridging system that was introduced more than 20 years ago and where three different linker molecules were involved: two biotinylated antibodies that bound the MLV gp and MHC class I or II proteins on the target cells respectively, and a bridging streptavidin molecule linking both antibodies. This led to the generation of a MLV that was capable of transducing MHC class I and II expressing cells (Roux, Jeanteur et al. 1989). Subsequently, two-protein molecular bridges have been exploited based on the avidin-biotin system. A recent example is provided by O'Leary et al. who used a detoxified recombinant form of the full-length botulinum neurotoxin, fused to core streptavidin that for its part was coupled to a biotinylated LV. This envelope gp construct endowed the LV particle with considerable neuron selectivity *in vitro* as well as *in vivo* after injection into the trachea (O'Leary, Ovsepian et al. 2011). Nowadays, alternative linkers such as ligand-receptor, chemical conjugations and monoclonal antibodies have been exploited to retarget LVs as well (Roux, Jeanteur et al. 1989; Boerger, Snitkovsky et al. 1999). For the latter, the E2 protein of the sindbis gp has been modified to contain the Fc-binding domain (ZZ domain) of protein A, making it possible to bind to a monoclonal antibody specific for a target molecule *via* its Fab antigen recognition end (Morizono, Xie et al. 2005). However, doubts are raised about the affinity of the adaptor-virus complex, as this may not be sufficient to prevent dissociation within the patient's blood. Moreover, complexity ascends as both the virion as the adaptor must be produced, purified and fully characterized for clinical approval. Another alternative possibility is to co-display a chimeric envelope gp together with a wild type gp such as VSV.G to enhance the transduction efficiency (Maurice, Verhoeven et al. 2002; Verhoeven, Dardalhon et al. 2003; Verhoeven, Wiznerowicz et al. 2005). However, this had also limited success due to partial loss of targeting specificity. Therefore, a final alternative is the usage of a mutated fusogenic but binding-defective envelope gp to mediate fusion upon binding by the chimeric gp. The group of Lin et al. co-expressed the MLV gp fused to soluble Fms-like tyrosine kinase 3 (Flt3)-ligand together with a binding-defective influenza hemagglutinin protein from the fowl plague virus rostock 34 (HAmu). When LVs were pseudotyped with both of these gps, Flt3-targeted transduction was enhanced when compared to LVs without HAmu and could be competed away by the addition of soluble Flt3-ligand (Lin, Kasahara et al. 2001). Another more straightforward strategy is the use of the E1/E2 heterodimer gp of sindbis virus as the fusion and binding functions are already separated over two different monomers. By mutating the binding E2 monomer, its binding property can be completely abolished. Therefore, this binding defective E2 protein forms an ideal scaffold for cell-specific antibody conjugation to confer specific tropism to an endless list of cell types such as P-gp-expressing melanoma progenitor cells and endothelial cells (Morizono, Xie et al. 2005; Pariente, Mao et al. 2008). A drawback is that they only induce fusion upon low pH. Therefore alternatives were explored such as the H and F protein of the measles virus, which induce fusion without the need for endocytosis (Earp, Delos et al. 2005; Funke, Schneider et al. 2009). This is exemplified by a study where a binding defective form of the H protein was fused to a CD20 specific scFv to pseudotype LVs. When these LVs were used to kill cells in culture, they selectively killed the CD20⁺ human lymphocytes in co-culture with CD20⁻ cells. This demonstrated the ability of these LVs to exclusively transfer a po-

tentially hazardous therapeutic protein into targeted cell populations with virtual absence of background transduction in non-target cells (Funke, Maisner et al. 2008). Meanwhile, a broad variety of surface antigens has been successfully targeted using this strategy (Blechacz and Russell 2008)

A fourth strategy to target LVs is based on two concepts: (1) the separation of binding and fusion functions over two distinct envelope molecules and (2) the ability of LVs to incorporate host cell proteins into their envelope as they bud from the plasmamembrane of their producer cells (Chandrashekrana, Gordon et al. 2004; Kueng, Leb et al. 2007). Chandrashekrana et al. reported on efficient and specific targeting to human cells expressing stem cell factor (SCF) receptor (c-kit) by an ecotropic gp pseudotyped LV which also displayed surface SCF. Another example is the overexpression of the HIV-1 derived primary receptor CD4 and fusogenic co-receptor CXCR4 or CCR5 on the membrane of producer cells. From these cells, LVs were generated that infect HIV-1 envelope gp expressing cells next to cells infected with HIV-1, enabling the development of novel antiviral therapy approaches (Somia, Miyoshi et al. 2000). Since the transduction efficiency was relatively low, LV co-enveloped with the HIV-1 cellular receptor CD4 and the E2 protein from sindbis virus were created. These turned out to have a higher infectivity level than in the former strategy (Lee, Dang et al. 2011). In another study the binding defective but fusogenic E1/E2 heterodimer was used to be co-displayed with a separate membrane bound anti-CD20 antibody in order to transduce exclusively CD20⁺ B cells (Lei, Joo et al. 2009). Today, numerous examples are found that apply this principle to target the following: immunoglobulin-expressing B cells, CD3⁺ T cells and CD117⁺ HSCs (Ziegler, Yang et al. 2008; Froelich, Ziegler et al. 2009; Yang, Joo et al. 2009). However, clinical applications with LVs displaying scFvs are hampered by lack of stability, size and immunogenicity leading to the development of neutralizing antibodies. To solve these problems, we developed the Nanobody (Nb) display technology (Goyvaerts, De Groeve et al. 2012). In this strategy, a fusogenic but binding-defective form of VSV.G (VSV.GS) (Zhang, Kutner et al. 2010) is co-displayed with a surface bound form of a cell-specific Nb to confer target binding (Figure 4). Some twenty years ago, Hamers-Casterman et al. discovered that part of the humoral response of Camelids is based on a unique repertoire of antibodies, which only consisted of two heavy chains (Hamers-Casterman, Atarhouch et al. 1993). The antigen binding part of these antibodies is composed of only one single variable region, termed VHH or Nb. These Nbs have unique characteristics and offer many advantages over scFvs to target LVs to specific cell types. These include (1) they are highly soluble, (2) they can refold after denaturation whilst retaining their binding capacity, (3) cloning and selection of antigen-specific Nbs obviate the need for construction and screening of large libraries, (4) as Nbs can be fused to other proteins, it is possible to present them on the cell membrane of a producer cell line, thus generating LVs that incorporate a cell-specific Nb in their envelope during budding. Using the Nb display technology, we demonstrated production of stable Nb pseudotyped LV stocks at high titers with a DC subtype specific transduction profile both *in vitro* as well as *in vivo* (Goyvaerts, De Groeve et al. 2012). As ligand specific Nbs can be generated to potentially every cell surface molecule, this technology will be applicable to target LVs to every cell type for which cell specific surface molecules are characterized (Gainkam, Huang et al. 2008; Vaneycken, Devoogdt et al. 2011).

The downside of the use of the above-described strategies is that they rely on the fusogenic capacity of a gp that is derived from viruses infectious to humans such as VSV, measles virus, sindbis virus and MLV. Their exposure to the complement or immune system, leading to anti-gp antibodies, might limit their clinical applicability. To surmount these obstacles, Frecha et al. pseudotyped LVs with a mutant fusogenic gp derived from an endogenous feline virus, named RD114. The mutant RD114 gp is an attractive candidate for *in vivo* use as it is resistant to degradation by the human complement. By co-displaying the early-acting-cytokine SCF together with mutant RD114 gp, human CD34⁺ HSCs could be targeted *in vivo* (Frecha, Fusil et al. 2011; Frecha, Costa et al. 2012). SCF was responsible for a slight and transient stimulation of the HSCs while preserving the ‘stemness’ of the targeted HSCs. In that way, the need for CD3/CD28 or cytokine pretreatment was obviated. Springfield et al. recently pseudotyped LVs with the H and F gps of the *Tupaia paramyxovirus* (TPMV), an animal virus without close human pathogenic relatives. Moreover, as this virus does not infect human cells, detargeting the H protein from its natural receptors is unnecessary. When LVs were pseudotyped with the TPMV envelope protein linked to an anti-CD20 single chain antibody, selective transduction of CD20⁺ cells, including quiescent primary human B cells, was reported (Enkirch, Kneissl et al. 2012).

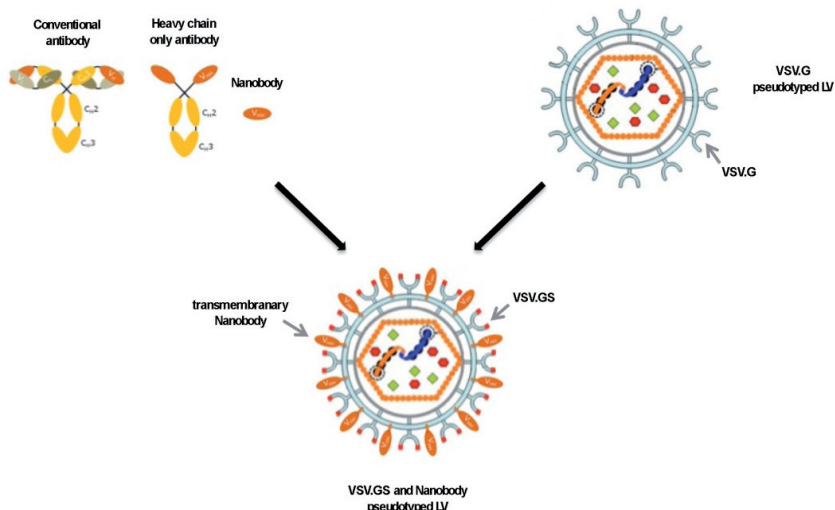


Figure 4. Principle of the Nb display technology. The Nb display technology is based on the fact that LVs need to bind and fuse with the membrane of a target cell for proper infection. While VSV.G accounts for both of these functions, we propose to separate these functions over two different molecules: (1) binding via a membrane bound Nb against the target cell of choice and (2) fusion via VSV.GS, which is a binding defective truncated version of VSV.G.

Recently an innovative alternative strategy has been described by Mannell et al. for site specific vascular gene delivery. In this case, the LVs were first coupled to magnetic nanoparti-

cles, which were in turn coupled to lipid microbubbles. LVs coupled to magnetic nanoparticles to target them to specific cell types *in vitro* using an external magnetic field has been described before. However, when these LV-nanoparticle constructs are considered for *in vivo* use, a sufficient magnetic moment is needed as the particles are subject to flow velocity within the blood vessels. As the magnetic moment is proportional to particle size, Mannell et al. coated the LV-nanoparticle constructs with magnetic microbubbles for enlargement. Upon intravenous delivery, the LV magnetic microbubbles were first trapped at the site of interest. Next ultrasound mediated destruction of the microbubbles resulted in fast release of the LVs at the site of interest with high transduction efficiency without the cost of higher cytotoxicity (Mannell, Pircher et al. 2012).

In conclusion, there seem to be some general prerequisites for successful transductional targeting of LVs: (1) use envelope gps with defined receptor binding sites, (2) abolish the natural recognition sites of the attachment gp, (3) separate fusion and attachment functions over two different molecules, (4) avoid the construction of large fusion constructs since their fusogenic capacities can be severely compromised and (5) avoid the use of immunogenic gps (Buchholz, Muhlebach et al. 2009).

5. Genomic targeting

Nowadays, LVs have become valuable tools for the treatment of several monogenic disorders such as hemophilia B, β -thalassemia and X-linked adrenoleukodystrophy (Cartier, Hacein-Bey-Abina et al. 2012; Payen, Colomb et al. 2012). However, the use of viral vectors that integrate their cargo into the genome of the host cell can trigger oncogenesis by insertional mutagenesis. This is exemplified by the incident where two out of 11 patients treated with a γ -retroviral vector to correct X-linked SCID, developed leukemia. This was caused by the γ -retroviral construct's tendency to insert into active genes, in this case the LMO-2 oncogene (Marshall 2002). Later on, using the same vector type to treat chronic granulomatous disease, genomic instability and myelodysplasia was observed (Stein, Ott et al. 2010). These incidents prompted substantial research into design, safety testing and optimization of integrating vectors. Thus far several measures have been taken to pose a reduced risk on insertional mutagenesis such as the development of SIN LVs containing a moderate cellular promoter (Modlich and Baum 2009; Montini, Cesana et al. 2009). Furthermore LVs are intrinsically less genotoxic than their retroviral counterparts (Montini, Cesana et al. 2006). Nevertheless, LVs have a higher transduction efficiency, which could counterbalance the reduced risk of mutagenic vector integration into the patient's genome. In addition, accumulating studies report the concept of LV-induced clonal dominance related to growth and/or survival advantage *e.g.* induced by vector integration and subsequent formation of aberrantly spliced mRNA forms (Fehse and Roeder 2008; Cavazzana-Calvo, Payen et al. 2010; Cesana, Sgualdino et al. 2012; Moiani, Paleari et al. 2012). In an extensive analysis to explore the effect of promoter-enhancer selection on efficacy and safety of LVs, no clear underlying mechanism could be provided for the observed. They concluded that other ill-defined risk factors must be involved for oncogenesis, including replicative stress (Ginn, Liao et al. 2010).

Finally, next to transcriptional activation of neighboring genes, also transcriptional shut off of the tg has been reported. This was due to chromatin remodeling at the site of insertion and cessation of the therapeutic effect (Stein, Ott et al. 2010).

Therefore additional strategies have been considered to reduce the side effects related to random insertion. The most straightforward strategy is to prevent integration of the proviral cargo by the use of IDLVs. These IDLVs are produced with a mutated integrase, which results in prevention of proviral integration and generation of increased levels of circular vector episomes within the infected cells. They appear to be safer with only a 0,1 to 2,3% chance that the episomal transcript gets integrated without a marked loss in effectiveness in terms of immune stimulatory potential of the IDLV-based vaccines (Vargas, Gusella et al. 2004; Philippe, Sarkis et al. 2006; Karwacz, Mukherjee et al. 2009; Wanisch and Yanez-Munoz 2009). However, as the lentiviral episomes lack replication signals, they are gradually lost by dilution in dividing cells and only stable in quiescent cells, which is undesirable for permanent correction of any genetic disorder. Furthermore also lower tg expression levels have been reported compared to integrative vectors (Bayer, Kantor et al. 2008). Therefore several alternative strategies have been brought forward to target the integrative process to a specific 'safe' genomic site.

In a first attempt, several groups tried to fuse a heterologous DNA binding domain directly to the integrase. Bushman et al. were the first to evaluate the activity of a hybrid, composed of the HIV-1 integrase and the lambda repressor. They reported on integration primarily near the lambda operator sites on the same face of the β -DNA helix (Bushman 1994). Later a model system was used where the integrase, derived from the avian sarcoma virus or HIV-1 respectively was fused to the *Escherichia coli* LexA repressor protein DNA binding domain (Katz, Merkel et al. 1996; Holmes-Son and Chow 2000). When this construct was packaged into the virion *in trans* either by replacing the original integrase gene or by cloning it adjacent to the HIV-1 accessory protein Vpr, they observed that this enhanced the use of integration sites adjacent to the *lexA* operators. In another study, the HIV-1 derived integrase was fused to a synthetic polydactyl zinc finger protein E2C, which binds specifically to a contiguous 18 bp E2C recognition site (Tan, Dong et al. 2006). Although in all studies clearly a higher preference for integration near the target sequence of choice was observed, this also implicated reduced DNA-binding specificity of the fusion protein with associated decrease of integration frequency of about 80 percent compared to viruses containing wild type integrase. Furthermore this strategy is also limited by the difficulty to incorporate the fusion protein into infectious virions (Michel, Yu et al. 2010).

Another strategy is targeting the integration away from genes using tethering domains linked to the host cell-encoded transcriptional co-activator lens epithelium-derived growth factor/p75 (LEDGF/p75), a cellular integrase binding protein. For example the LEDGF/p75 chromatin interaction-binding domain has been replaced with CBX1, which binds histone H3 di- or trimethylated on K9. Subsequently proviral integration was directed to pericentric heterochromatin and intergenic regions (Llano, Vanegas et al. 2006; Ferris, Wu et al. 2010; Gijssbers, Ronen et al. 2010; Silvers, Smith et al. 2010). As this requires engineering of a host cell protein, it is not feasible for clinical applications at the present stage (Izmiryan, Basma-

ciogullari et al. 2011). Site-specific proviral integration can also be mediated by the use of site-specific recombinases. The best known are derived from the lambda integrase family of enzymes and include the bacteriophage P1 Cre recombinase, bacteriophage lambda integrase, the yeast FLP recombinase and bacterial XerCD recombinase. They catalyze site specific recombination by a transient DNA-protein covalent linkage that brings two specific DNA repeats together (Van Duyne 2001). Depending on the orientation of the DNA repeats, the DNA segment will either be excised or inverted when in the same or opposite orientation respectively (Figure 5A, adapted from http://www.ruf.rice.edu/~rur/issue1_files/norman.html). The Cre-loxP system has been developed for gene studies to conditionally knock out a target gene in a cell- or tissue specific manner to overcome embryonic lethality due to permanent inactivation of the target gene in an early developmental stage (Ray, Fagan et al. 2000). This system is based on two palindromic loxP sites of 34 bp that flank the gene of interest. Although these loxP sites are prevalent in the genomes of bacteriophages, they are absent in the mouse genome where they have to be introduced by targeted mutagenesis (Kos 2004). Throughout the human genome, however, loxP-like sequences or pseudo-loxP sites are present that can be recognized by either wild-type Cre or Cre variants. This last feature enables site-specific insertion of a gene in a defined loxP site in the human genome if a Cre recombinase is provided in *cis* or *trans*. Michel et al. evaluated the feasibility of combining the Cre-loxP system for gene targeting with the versatile gene delivery system of LVs for site-specific gene insertion in human cell lines. They transduced a loxP site containing cell line with a LV containing Cre recombinase in *trans* as a fusion protein to the HIV accessory protein Vpr. Moreover the LV contained a cassette containing a loxP site followed by the neomycin resistant gene, inserted in the U3 region of the 3'LTR. Upon reverse transcription, the loxP-neo sequence would appear in both LTRs, thereby providing a substrate for recombination that could be catalyzed by the virion-associated Vpr-Cre. Upon this recombination step, a circular product was produced that was on his turn inserted into the loxP site of the cell line, again catalyzed by virion-associated Vpr-Cre. Another example is provided by the group of Jiang et al. who demonstrated a selective inhibitory effect on the lens epithelial cells and not the retinal pigment epithelial cells (Jiang, Lu et al. 2011). Therefore they used an enhanced Cre/loxP system with a LV expressing Cre under the control of the lens-specific promoter LEP503 in combination with another LV that contained a stiffer sequence encoding eGFP with a functional polyadenylation signal between two loxP sites, followed by the HSV-TK gene, both under the control of the human phosphoglycerate kinase promoter. Expression of the downstream HSV-TK was activated by co-expression of Cre under the control of the lens-specific promoter LEP503. Although this technology allows site-specific tg insertion, there are only a limited amount of pseudo-loxP sites in the human genome and even none in the mouse genome, which makes this technique unusable for fundamental research in laboratory animals. Furthermore, two recombination events are required which has a major impact on its efficiency.

A recent strategy makes use of site-specific endonucleases to target the tg to neutral 'safe harbor' genome regions or stimulate the process of homologous recombination for gene repair (Fischer, Hacein-Bey-Abina et al. 2011). Endonucleases induce site-specific ds breaks that can be repaired by homology-directed repair, a form of homologous recombination that

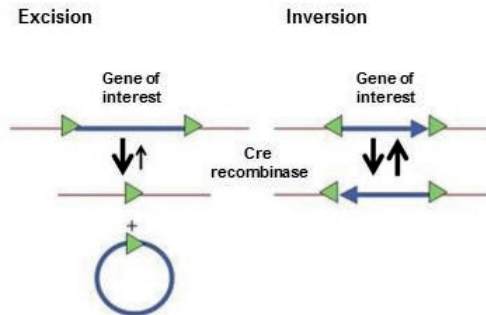
uses a copy of the genetic information from the broken DNA molecule. When the latter is provided by the same or another LV, this copy will be used to repair the ds break (Urnov, Miller et al. 2005; O'Driscoll and Jeggo 2006). The advantage of gene repair/correction is that both function and expression of the affected gene are restored while the risk associated with random vector integration is avoided. Besides the advantage of the reduced risk for insertional mutagenesis, this strategy is also used to target genes in order to knock them down or replace them with another gene by homologous recombination. The disadvantage is that the nuclease coding sequences are expressed for several days, which is not optimal for translation to the clinic due to the background off-target generation of dsDNA breaks.

One possibility is the use of the zinc finger nuclease strategy. For this, the Cys2His2 class of zinc finger DNA binding domains is engineered to recognize a DNA sequence of interest, fused to the nuclease domain of the FokI type II restriction endonuclease to yield a highly specific zinc finger (Figure 5 B, adapted from <http://biol1020-2011-2.blogspot.be/2011/09/zinc-finger-nucleases-zfn-emerging.html>) (Kim, Cha et al. 1996; Pabo, Peisach et al. 2001). When two different zinc fingers are designed to bind the same sequence of interest in the opposite orientation, this will allow dimerization of the FokI domains which leads to a zinc finger induced dsDNA break (Bitinaite, Wah et al. 1998). Various strategies have been developed to engineer the Cys2His2 zinc fingers in order to bind a specific sequence either by modular assembly or by selection strategies using phage display or a cellular selection system. Naldini et al. evaluated the use of zinc finger nucleases in combination with an IDLV for gene editing. Therefore they co-transduced several cell lines with three different IDLVs, one encoding the donor sequence and two encoding the two zinc fingers (Lombardo, Genovese et al. 2007). A few years later they also used this strategy to assess zinc finger specificity genome-wide by comprehensively mapping the locations of the IDLV integration sites in cells co-transduced with GFP and zinc finger encoding LVs (Gabriel, Lombardo et al. 2011). They observed a very high efficiency and specificity, yet a measurable rate of vector integration at unidentified sites occurred with this approach, which is the sum of zinc finger mediated and background levels of IDLV integration. Moreover co-transduction with three different LVs may be a rate-limiting step in this system. Therefore the use of a single construct to express the zinc fingers and deliver the donor tg must be evaluated, especially for less permissive cells such as hematopoietic progenitors.

Another way to target the proviral genome is by the provision of a vector-associated meganuclease encoded by a separate vector or supplied as a protein within the viral particle. (Izmiryan, Basmaciogullari et al. 2011). For the latter, Izmiryan et al. fused the prototypic meganuclease I-SceI from yeast to Vpr. This avoided the potentially toxic sustained expression of the introduced endonuclease. IDLVs encoding the donor sequence and containing the meganuclease-SceI fusion construct were tested in reporter cells in which targeting events were scored by the repair of a puromycin resistance gene. They reported a two-fold higher frequency of the expected recombination event when the nuclease was delivered as a protein rather than encoded by a separate vector and therefore improved both the safety and efficacy of this LV-based gene targeting system. In conclusion, although the field of ge-

nomic targeting is relatively new for LV-based gene therapy, it opens a tremendous amount of new possibilities.

A) Cre loxP recombination



B) The zinc finger nuclease complex

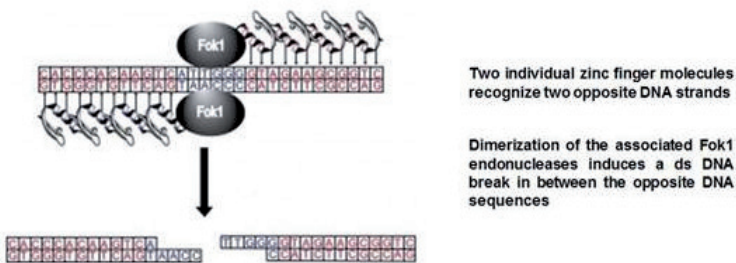


Figure 5. Schematic representation of targeted genome modification by the Cre-loxP system (A) or by the zinc-finger nuclease complex (B). Cre recombinases recognize specific loxP sites in the genome, bind them and bring them together. Depending on their orientation this leads to excision (same orientation) or inversion (opposite orientation) of the sequence flanked by the two loxP sites (A). Two individual zinc finger molecules each recognize a 9 to 18 bp DNA sequence using between three and six individual zinc finger repeats that bind the major groove of DNA. The DNA sequences are non-palindromic DNA sites located respectively up- and downstream of the intended cleavage site, which is mostly about 5-7 bp long. If the zinc finger domains are perfectly specific for their intended target site then even a pair of three-finger ZFNs that recognize a total of 18 bp can theoretically target a single locus in a mammalian genome. Next, the associated FokI nucleases dimerize and induce a double stranded break which can be restored by either non-homologous end-joining or homology-directed repair, which faithfully restores the original sequence by copying it from the sister chromatid or using the homologous sequence provided by a LV (B).

6. Concluding remarks

LVs have proven to be efficient vehicles to deliver one or more tgs to any cell type of choice, which has led to a promising list of therapeutic applications. As the demand for experimentation in gene delivery to specific cell types increases, technologies that precisely target LV-based gene expression will become more important for research and clinical applications. Four main groups of strategies with their own possibilities as well as difficulties have been developed so far. Self-evidently, further optimization and fine-tuning of these strategies is a necessity to fulfill the expectations for targeted LV delivery *in vivo*. In addition to extra optimization steps, combinations of two or more of these strategies can also lead to an overall more selective, efficient and most importantly, safe LV system. Several attempts to combine the different strategies have been reported (Brown, Cantore et al. 2007; Pariente, Morizono et al. 2007; Escors and Breckpot 2011). Pariente et al. for example, reported on a LV that was transductionally targeted to prostate cancer bone metastases by a modified sindbis virus envelope that interacts with PSMA and transcriptionally targeted with a prostate cell specific promoter. This dual-targeted LV enhanced specificity to prostate cancer bone metastases after systemic delivery with respect to individual transcriptional or transductional targeting. As the developed targeting strategies already resulted in a major step forward for LV-based gene therapy, their potential will most likely be more exploited in the future, paving the way towards an all-embracing LV-based tg vehicle for the gene therapeutic field.

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Vectors for Highly Efficient and Neuron-Specific Retrograde Gene Transfer for Gene Therapy of Neurological Diseases

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Additional information is available at the end of the chapter

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1. Introduction

Viral vectors have been widely used to deliver several therapeutic genes in the clinical approach of gene therapy. The lentiviral vector permits stable and efficient gene transfer into non-dividing cells in the central nervous system of neurological and neurodegenerative diseases (Deeks, et al., 2002; Mavilo, et al., 2006; Rossi et al., 2007; Ciceri, et al., 2009; Naldini, 2011). Moreover, long-term expression of delivered gene attributed to genome integration has an advantage not only for clinical application, but also for gene therapy trials in animal models (Naldini et al., 1996; Reiser et al., 1996; Mochizuki et al., 1998; Mitrophanous et al., 1999; Wong et al., 2006; Lundberg et al., 2008). Among many lentiviral vector systems, the most familiar is the human immunodeficiency virus type-1 (HIV-1)-based vector of which molecular biological property has been extensively studied (Rabson and Martin, 1985; Joshi and Joshi, 1996; Nielsen et al., 2005; Pluta and Kacprzak, 2009).

Axonal transport in the retrograde direction, as observed in the case of some viral vectors, has a considerable advantage for transferring genes into neuronal cell bodies situated in regions remote from the injection sites of the vectors (see Fig.1). These viral vectors, for example, injected into the striatum, transfer the genes via retrograde transport into nigrostriatal dopaminergic neurons, which are the major target for gene therapy of Parkinson's disease (Zheng et al., 2005; Barkats et al., 2006). Intramuscular injection of the vectors also delivers retrogradely the genes into motor neurons that are the target for gene therapy of motor neuron diseases (Baumgartner & Shine, 1998; Perrelet et al., 2000; Mazarakis et al., 2001; Sakamoto et al., 2003; Azzouz et al., 2004).

In our previous study, we generated an HIV-1-based vector pseudotyped with a variant of rabies virus glycoprotein (RV-G) gene and tested gene transfer through retrograde axonal transport into several brain regions (Kato et al., 2007). Although this pseudotyped vector showed gene transfer through retrograde transport in the rodent and nonhuman primate brains, higher titer stocks of the vector was required for the application of gene therapy trials. To enhance the efficiency of retrograde gene transfer, we subsequently developed a novel type of lentiviral vector that shows highly efficient retrograde gene transfer (HiRet) by pseudotyping an HIV-1-based vector with fusion glycoprotein B type (FuG-B) composed of parts of RV-G and vesicular stomatitis virus glycoprotein (VSV-G) (Kato et al., 2011a,b).

More recently, we developed another vector system for neuron-specific retrograde gene transfer (NeuRet) by pseudotyping the HIV-1-based vector with fusion glycoprotein C type (FuG-C) composed of a different set of parts of RV-G and VSV-G (Kato et al., 2011c). Interestingly, the NeuRet vector shows high efficiency of retrograde gene transfer into various neuronal populations, whereas it remarkably reduces gene transduction into dividing cells including glial and neural stem/progenitor cells around the vector injection sites. One significant issue on the therapeutic use of lentiviral vectors is transgene integration into the host genome in dividing cells, which may lead to tumorigenesis by altering the expression of proto-oncogenes adjacent to the integration sites (De Palma et al., 2005; Themis et al., 2005; Montini et al., 2006). In this context, the NeuRet vector can reduce the risk of vector transduction into dividing cells in the brain and improve the safety of future gene therapy trials for neurological and neurodegenerative disorders.

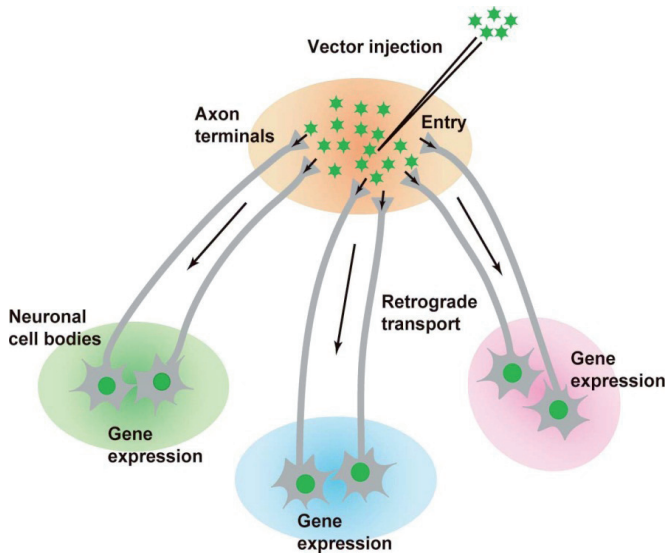


Figure 1. Gene transfer process through retrograde axonal transport.

The viral vectors enter nerve terminals and are retrogradely transported through axons into neuronal cell bodies, resulting in the induction of transgene expression.

In this chapter, we recapitulate gene transduction property of the HiRet and NeuRet vectors, and then describe the application of the NeuRet vector for retrograde gene transfer into the nigrostriatal dopamine system in nonhuman primates.

2. Gene transduction property of HiRet and NeuRet vectors

2.1. HiRet vector

The HiRet vector is a pseudotype of the HIV-1 lentiviral vector with FuG-B, which is composed of the extracellular and transmembrane domains of RV-G (challenged virus standard strain) and the cytoplasmic domain of VSV-G (Fig. 2A) (Kato et al., 2011a). When the HiRet vector encoding green fluorescent protein (GFP) was injected into the dorsal striatum of mice, we observed high efficiency of retrograde gene transfer into the brain regions innervating the striatum, including the primary motor cortex (M1), primary somatosensory cortex (S1), parafascicular nucleus (PF) in the thalamus, and substantia nigra pars compacta (SNc) in the ventral midbrain (Fig. 2B). The extent of gene transfer efficiency increased compared with that of the RV-G pseudotype, ranging from 8- to 14-folds dependent on the neural pathways. The high efficiency of gene transfer was also detected in the brain regions that project to the nucleus accumbens or medial prefrontal cortex in mice. In addition, we observed gene transduction of the HiRet vector into glial cells (~75%) and a small number of neuronal cells (~20%) in the striatum around the injection sites (Fig. 2C). Recently, we created a variant of FuG-B (termed FuG-B2), in which the extracellular and transmembrane domains of RV-G derived from the challenged virus standard strain was exchanged with the counterparts of Pasteur virus strain, and the vector pseudotyped with FuG-B2 exhibited a further increase in the retrograde gene transfer efficiency in the rodent brain (Kato et al., 2011b). More recently, Carpentier et al. (2012) reported the increased pseudotyping efficiency of an HIV-1 vector by a chimeric envelope glycoprotein composed of RV-G and VSV-G domains, which corresponds to our FuG-B.

The host range of lentiviral vectors is altered by pseudotyping with different envelope glycoproteins (Cronin et al., 2005). Therefore, the possibility arises that some mutations in RV-G shift the efficiency of gene transduction or host cell specificity of the pseudotyped vector. Indeed, substitution of the cytoplasmic domain of RV-G with the corresponding part of the VSV-G enhanced the efficiency of retrograde gene transfer. The cytoplasmic domain differs in length between RV-G (44 amino acids) and VSV-G (29 amino acids), but their amino acid sequences do not show any particular homology (Rose et al., 1982). It appears that the cytoplasmic domain is involved in the mechanism underlying vector entry into synaptic terminals or the transduction level of the vector, resulting in enhanced retrograde gene transfer.

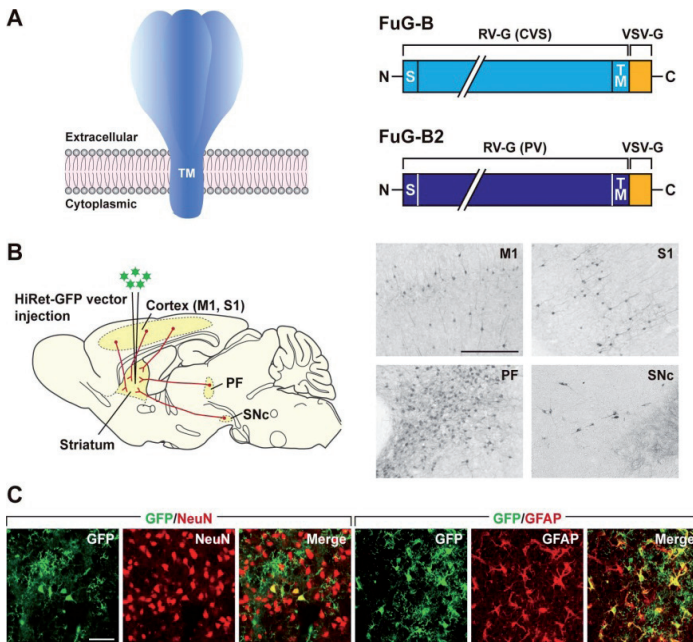


Figure 2. Gene transfer by HiRet vector. **(A)** Fusion envelope glycoprotein. The structure of viral envelope glycoprotein is schematically illustrated in the left panel. FuG-B is composed of the extracellular and transmembrane (TM) domains of RV-G derived from the challenge virus standard (CVS) strain fused to the cytoplasmic domain of VSV-G. In FuG-B2, the RV-G domains are exchanged by the counterparts of RV-G derived from Pasteur virus (PV) strain. S, signal peptide. **(B)** Gene transfer through retrograde transport. The HiRet vector pseudotyped with FuG-B, encoding GFP transgene was injected into the mouse striatum. Four weeks later, sections were processed for GFP immunostaining (right panel). GFP expression can be seen in the brain regions innervating the striatum, including the M1, S1, PF, and SNc. **(C)** Gene transduction around the injection sites. Sections through the striatum were stained by double immunofluorescence histochemistry for GFP/NeuN or for GFP/glial fibrillary acidic protein (GFAP). Scale bars: 50 μ m. (Data from Kato et al., 2011a)

2.2. NeuRet vector

The NeuRet vector is another pseudotype of the HIV-1 lentiviral vector with FuG-C, which is composed of the N-terminal segment of the extracellular domain (439 amino acids) of RV-G and the C-terminal segment of the extracellular domain (16 amino acids) and transmembrane/cytoplasmic domains of VSV-G (Fig. 3A) (Kato et al., 2011c). After injection of the NeuRet vector encoding GFP transgene into the mouse striatum, we found enhanced retrograde gene transfer into the brain regions innervating the striatum, such as the M1, S1, PF, and SNc (Fig. 3B). The efficiency of gene transfer of the NeuRet vector was slightly different with that of the HiRet vector (FuG-B2 pseudo type), depending on the neural pathways (see a review by Kato et al. 2012). In addition, we tested gene transduction of the NeuRet vector surrounding the injection sites. Although the NeuRet vector transduced only a small num-

ber of striatal neuronal cells (~6%), its transduction level into striatal glial cells was quite low (~0.3%) (Fig. 3C). The property of gene transduction of the NeuRet vector around the injection sites was quite different from that of the HiRet vector, and in particular, the transduction of glial cells was largely declined in the NeuRet vector. Furthermore, when the NeuRet vector was injected into the subventricular zone, gene transduction of the vector into neural stem/progenitor cells was also inefficient.

FuG-C pseudotyping of the NeuRet vector enhanced the efficiency of retrograde gene transfer into various neuronal populations, whereas it caused less efficiency of gene transduction into glial and neural stem/progenitor cells. The N-terminal segment of the RV-G extracellular domain of 439 amino acids appears to be involved in the retrograde gene transfer, probably by promoting the interaction with synaptic terminals required for retrograde transport. Actually, amino acid residues essential for rabies virus virulence are reported to exist in the RV-G-derived extracellular domain used for FuG-C construction (Prehaud et al., 1988; Coulon et al., 1998). In contrast, pseudotyping with FuG-B (FuG-B2) and FuG-C generates a marked difference in gene transduction into glial and neural stem/progenitor cells around the injection areas. This difference suggests that the C-terminal part of 16 amino acids in the extracellular domain of envelope glycoproteins may be implicated in determining the host cell specificity of vector transduction, and that this C-terminal part may contribute to the interaction with glial and neural stem/progenitor cells.

For gene therapy trials with lentiviral vectors, there is a significant issue that vector insertion into the host genome may lead to tumorigenesis by altering the expression of cellular oncogenes surrounding the integration sites (De Palma et al., 2005; Themis et al., 2005; Montini et al., 2006). One useful approach to protect this issue is to restrict vector transduction to neuronal cells. The NeuRet vector system provides a useful approach for gene therapy trials for neurological diseases through enhanced retrograde gene transfer and improves the safety of gene therapy by profoundly suppressing the efficacy of gene transduction into dividing cells in the brain.

3. Retrograde gene delivery into monkey nigrostriatal pathway by NeuRet vector

The nigrostriatal dopamine system is a major target for gene therapy of Parkinson's disease. The availability of the HiRet vector for gene transfer via retrograde transport into the nigrostriatal dopamine system in nonhuman primates was described in our previous review (Kato et al., 2011d). To verify the capability of the NeuRet vector for efficient retrograde gene transfer into the nigrostriatal pathway, we injected the NeuRetvector encoding the GFP transgene into the striatum (caudate nucleus and putamen) of crab-eating monkeys (Fig. 4A). Intrastratial injection of the NeuRet vector produced a larger number of GFP-positive neurons in the SNc (Fig. 4B). These positive signals were in register with immunostaining for tyrosine hydroxylase, a marker of dopaminergic neurons (Fig. 4C), indicating the transgene expression in the nigrostriatal dopaminergic neurons. In addition, we assessed the

property of gene transduction with the NeuRet vector around the injection sites in the monkey striatum. The vector displayed a low level of gene transfer into neuronal cell bodies (~13%), and the level of vector transduction into glial cells was also quite low in the monkey striatum (~0.6%) (Fig. 4D). The pattern of gene transduction around the injection sites was similar to that obtained from the analysis of the mouse brain sections. Therefore, the NeuRet vector mediates enhanced retrograde gene transfer, whereas it reduces the gene transfer into glial cells around the injection areas in both rodent and monkey brains.

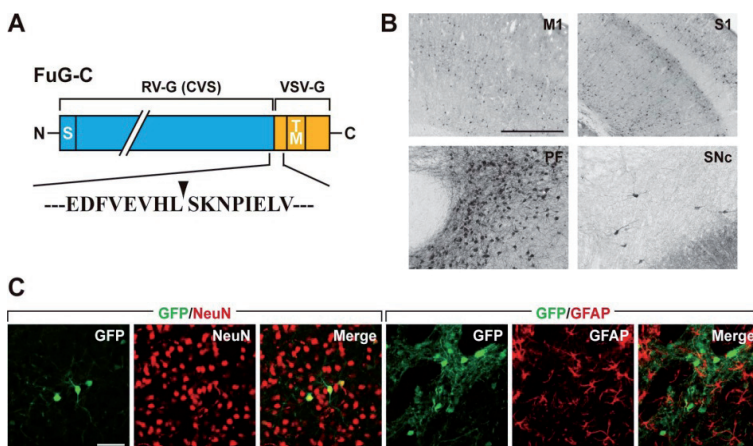


Figure 3. Gene delivery by NeuRet vector. **(A)** Structure of fusion envelope glycoprotein. FuG-C is composed of the N-terminal segment of the extracellular domain of RV-G and the C-terminal segment of the extracellular domain and the transmembrane(TM)/cytoplasmic domains of VSV-G. Amino acid sequences around the junction between the RV-G and VSV-G segments are shown. S, signal peptide. **(B)** Gene transfer through retrograde transport. The NeuRet vector encoding GFP transgene was injected into the mouse striatum, and four weeks later sections were processed for GFP immunostaining. GFP expression can be visualized in the M1, S1, PF, and SNc. **(C)** Gene transduction around the injection sites. Sections through the striatum were stained by double immunofluorescence histochemistry for GFP/NeuN or for GFP/glial fibrillary acidic protein (GFAP). Scale bars: 50 μ m. (Data from Kato et al., 2011c)

The NeuRet vector system successfully achieved efficient gene transfer through retrograde transport into the nigrostriatal dopaminergic neurons in nonhuman primates. Our vector system will provide a powerful strategy for gene therapy of Parkinson's disease with enhanced retrograde gene transfer in the near future. This system will improve the safety of gene therapy by reducing the risk of gene transduction into proliferating cells (glial and neural stem/progenitor cells) in the brain.

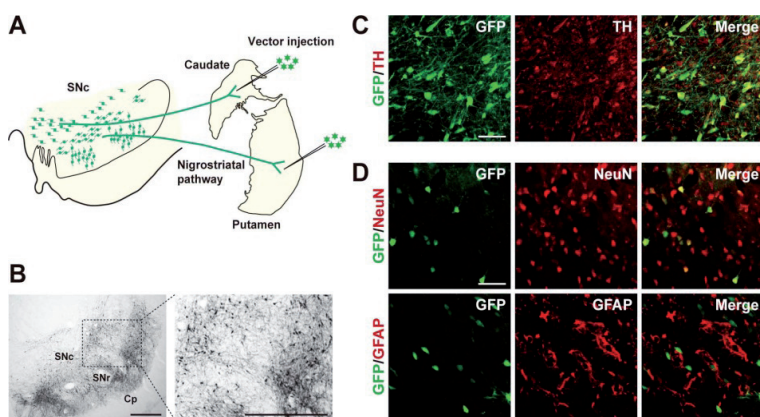


Figure 4. Transgene expression in the nigrostriatal dopamine system by NeuRet vector injection into the monkey striatum. (A) Gene transfer through retrograde transport after intrastratial injection. The NeuRet vector encoding GFP transgene was stereotactically injected into the caudate nucleus and the putamen, and histological analysis was performed on the brains fixed at the 4-week postinjection period. (B) GFP immunostaining in the SNc, CP, cerebral peduncle; SNr, substantia nigra pars reticulata. (C) Double immunofluorescence staining for GFP and tyrosine hydroxylase (TH) in the SNc. (D) Double immunofluorescence staining for GFP/NeuN or GFP/glial fibrillary acidic protein (GFAP) in the striatum. Scale bars: 500 μ m (B), and 50 μ m (C, D). (Data from Kato et al., 2011c)

4. Conclusion

In this chapter, we mentioned the gene transduction property of the HiRet and NeuRet vectors pseudotyped with different fusion envelope glycoproteins. These two vectors showed the enhancement in gene transfer through retrograde axonal transport into various neuronal populations in both rodent and nonhuman primate brains. The HiRet vector transduced prominently glial cells around the injection sites, whereas gene transduction of the NeuRet vector into glial cells was much less efficient. The transduction level of the NeuRet vector into neural stem/progenitor cells was also low. The variation in the structure of envelope glycoproteins shifted the efficiency of retrograde gene transfer and the preference of host range. In addition, we described the application of the NeuRet vector for retrograde gene transfer into the nigrostriatal dopamine system of monkeys. The NeuRet vector, together with the HiRet vector, will offer a promising technology for gene therapy of neurological diseases through enhanced retrograde gene transfer. In particular, the NeuRet vector system will improve the safety of gene therapy by greatly suppressing the risk of gene transduction into dividing cells in the central nervous system.

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Retroviral Genotoxicity

Dustin T. Rae and Grant D. Trobridge

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52530>

1. Introduction

Gene therapies have enormous potential to cure human disease. In recent years, hematopoietic stem cell (HSC) gene therapy has advanced tremendously, due in part to years of intense research to develop effective vectors and efficient ex vivo transduction protocols. In early clinical trials, inefficient gene transfer resulted in either a lack of therapeutic benefit or short-lived therapeutic benefit [1-3]. Advances in preclinical animal models, led to improved gene transfer in human clinical trials, where long-term efficacy has now been achieved. HSC gene therapy has been used to correct several monoallelic genetic diseases [4], such as X-linked severe combined immune-deficiency (SCID X-1) [5], chronic granulomatous disease (CGD) [6-8], adenine deaminase deficiency (ADA-SCID) [9-12], Wiskott-Aldrich syndrome [13-14], and X-linked adrenoleukodysrophy [15,16]. Recently HSC gene therapy has also been used to treat glioblastoma [17], X-linked hyper-immunoglobulin M syndrome (HIGM), and familial haemophagocyticlymphohistiocytosis syndrome (HLH) [18]. These successes are in large part due to advances in ex vivo transduction protocols and improvements with recombinant vector technologies. The French SCID-X1 HSC gene therapy trial marked a major turning point in the field when nine of the ten patients treated exhibited therapeutic benefit. However, following this exciting achievement the field was dealt a major setback when it was initially reported, that two patients from the study had developed vector-mediated leukemia resulting from the treatment [19]. This was the first vector-mediated malignancy reported in a HSC gene therapy clinical trial. Four boys ultimately developed leukemia as a side effect of the gene therapy procedure [5]. Three of the four boys were successfully treated with chemotherapy, but one patient died due to vector-mediated T cell leukemia. In these patients, vector-mediated dysregulation of host genes led to leukemia, and this unwanted adverse side effect is currently a major challenge for HSC gene therapy. The effect of the integrated viral vector on host gene expression resulting in an altered phenotype is known as genotoxicity.

Genotoxicity is a result of retroviral mediated delivery of the integrated form of the retroviral vector genome known as the vector provirus into the host genome. Integration of the vector provirus into a host chromosome, by definition, alters the host DNA. In cases where a retrovirus or retroviral vector provirus has dysregulated host gene expression, insertional mutagenesis is said to have occurred. However, it is important to remember that provirus integration always results in mutation of the host genome, regardless of whether the vector provirus exerts an effect on host gene expression. The oncogenic properties of replication-competent retroviruses were well known prior to the development of retroviral vectors for gene therapy. However, vectors that are used in gene therapy have been engineered so that they do not have the ability to replicate, only to insert their genome into a target cell. These vectors are thus referred to as replication-incompetent. In numerous preclinical and clinical studies conducted prior to the SCID-X1 trial, malignancies were not observed when using replication-incompetent vector systems [20]. It was therefore assumed that the potential for malignant transformation from a replication-incompetent vector was very low. Unfortunately, it has now been clearly shown in the French SCID-X1 trial and in subsequent HSC gene therapy trials, that genotoxicity is indeed a problem for replication-incompetent vectors. Here we review the mechanisms of vector-mediated genotoxicity in HSC gene therapy and describe efforts in the field to reduce genotoxicity which is currently a major challenge in the field [21, 22].

2. Why integrating vectors are used for HSC gene therapy

Why use integrating vectors for HSC gene therapy if we know that retroviral vectors mutagenize the genome and therefore carry a risk to induce genotoxicity? The answer is that provirus integration to HSCs is currently the only way to efficiently and stably deliver transgenes to the billions of mature blood cells produced every day in the body. Our mature blood cells are generated from a relatively small pool of self-renewing long term-repopulating HSCs in the bone marrow through a process known as hematopoiesis (Figure 1). During hematopoiesis, long-term repopulating stem cells provide lifelong supplies of the mature cells of each blood cell lineage via massive expansion of transit-amplifying cells that include multi-potent and lineage restricted progenitors. By permanently modifying a long term repopulating HSC via proviral integration into the HSC genome we can ensure that all progeny produced from these gene-modified cells will inherit the transgene during mitosis. Thus, the mature blood cells that arise during hematopoiesis from gene-modified HSCs and their daughter transit amplifying cells all inherit the transgene. Using retroviral vectors to efficiently deliver a therapeutic transgene via integration of the vector provirus into a HSC is currently the only effective approach for HSC gene therapy. While there have been reports of some success with adenovirus and other non-integrating approaches in small animal models, to date only integrating vectors have been used successfully for HSC gene therapy in large animal models and in clinical trials.

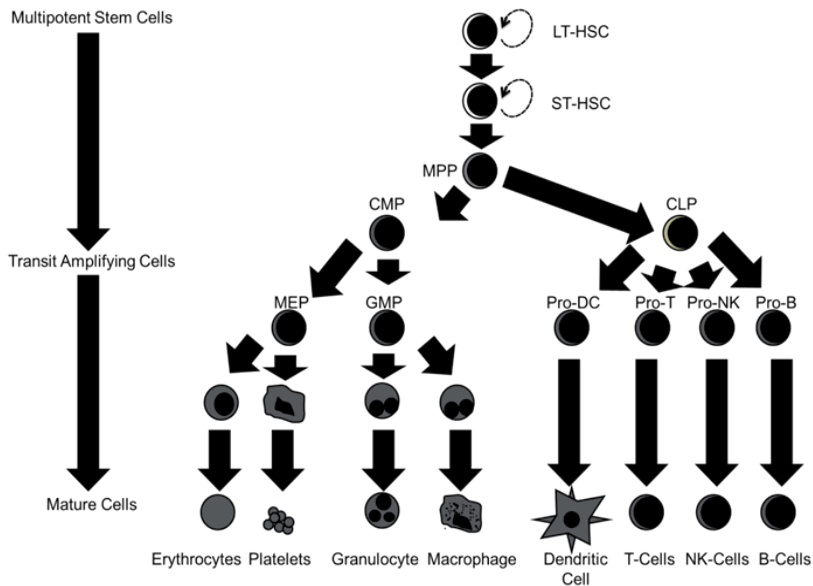


Figure 1. Human Hematopoiesis. Long-term-hematopoietic stem cells (LT-HSCs) are a self-renewing population of stem cells that reconstitute the blood system throughout the entirety of our life span. Short-term-HSCs, reconstitute our blood system for only limited periods. The short-term-HSCs differentiate into multipotent progenitors (MPPs), which have the ability to differentiate into several transit amplifying cell lineages. Common lymphoid progenitors (CLPs) differentiate into (Pro- Dendritic Cell, Pro-T, Pro-NK, and Pro-B) lymphoid progenitor cells. Finally, these progenitors give rise to the mature lymphoid class cells of the blood system (T- lymphocytes, B-lymphocytes, and natural killer (NK) cells). Common myeloid progenitors (CMPs), give rise to granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) that differentiate into :(macrophages, granulocytes, megakaryocytes, and erythroid) myeloid class progenitors. Finally, these progenitors give rise to the mature myeloid class cells of the blood system.

3. Retroviruses as insertional mutagens

We now know that the use of retroviral vectors for HSC gene therapy, though highly efficient, can dysregulate host genes near the vector provirus and ultimately lead to malignant transformation. The ability of replicating retroviruses to cause tumorigenesis is well established. In 1911, Peyton Rous showed that a sarcoma growing on a domestic chicken could be transferred to another chicken by exposing the healthy bird to a cell-free filtrate [23]. This filterable agent is now known to be the Rous sarcoma retrovirus. Since this report, many retroviruses have been discovered that cause diverse malignancies. There are several mechanisms whereby retroviruses can cause malignancy. Varmus et al. showed that acutely transforming onco-retroviruses capture and deliver cellular oncogenes, which allow these viruses to efficiently, convert target cells into a malignant phenotype [24]. It is important to

note that oncogene capture does not occur at a detectable frequency with current replication-incompetent vectors used in gene therapy. Yet several mechanisms remain for cellular transformation from replication-incompetent retroviral vector proviruses (Figure 2). Despite the risks associated with malignant transformation from retroviral vectors via insertional mutagenesis, for several severe hematopoietic diseases the therapeutic benefit of HSC gene therapy outweighs the risks. Currently, major efforts are underway to further our understanding of genotoxic events and to improve vector safety by reducing their genotoxic potential [18, 25, 26].

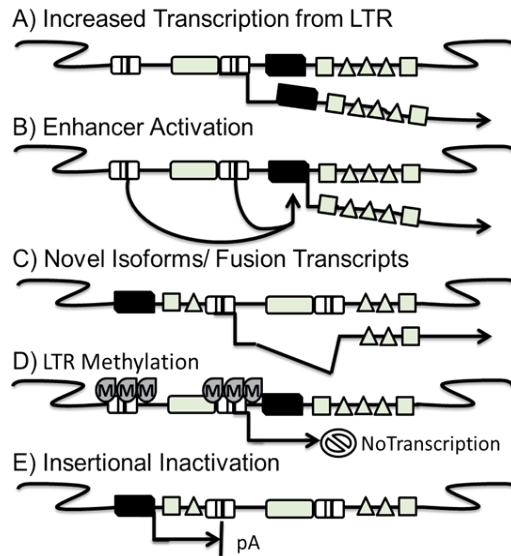


Figure 2. Mechanisms of Retroviral Mutagenesis. The black boxes represent promoters, and grey squares represent exons. A) The proviral 3' LTR can drive transcription of nearby cellular gene at an increased rate. B) Proviral LTR enhancers can activate a nearby promoter, increasing transcription of cellular genes. C) Transcription from 5' LTR in conjunction with proviral cryptic splice sites creates novel isoforms and fusion transcripts of both cellular and viral genes. D) Proviral LTR methylation, induces epigenetic changes, silencing proviral genes and nearby cellular genes. E) Proviral integration can disrupt cellular gene expression by causing premature polyadenylation (pA) signaling.

4. Overview of ex vivo HSC gene therapy

It is important when studying genotoxicity to consider how the target cells are manipulated during the gene transfer process. HSC gene therapy is conceptually straightforward but requires culture of stem cells under the appropriate ex vivo conditions. A patient's cells are collected and enriched for repopulating stem cells using the CD34 marker. The CD34 protein is a member of the sialomucin family, and is expressed in early HSCs [27]. The CD34

marker allows for rapid enrichment of HSCs, typically via column enrichment using CD34 antibody-conjugated magnetic beads. CD34-enriched cells that include repopulating HSCs are then exposed to the vector containing the therapeutic gene in an ex vivo transduction process. Following ex vivo transduction, gene modified cells must be infused into the patient. Correction of the disease phenotype will occur if enough gene-modified repopulating cells engraft. Engraftment requires that gene modified cells survive, home to the bone marrow, and proliferate sufficiently to repopulate the blood system.

Early preclinical HSC gene therapy studies in mice demonstrated high gene transfer rates. However, early clinical trials using similar approaches and culture conditions had inefficient gene transfer [28, 29]. Large animal models such as the dog and non-human primates more accurately model human HSC gene therapy and have since been used to establish the conditions for efficient ex vivo transduction [30]. More effective ex vivo gene protocols were developed, that resulted in higher gene transfer efficiencies while maintaining efficient engraftment. These improvements included defining cytokine support and the extracellular matrix CH-296 fibronectin fragment [5, 31, 32]. Together these advances contributed to the success of HSC gene therapy clinical trials such as the French SCID-X1 trial, and now efficient transduction of human CD34+ cells can be routinely achieved using retroviral vectors. These improved gene transfer efficiencies also factored into the observed genotoxicity. For example, two patients in the French SCID-X1 trials were estimated to have a total of 4.3×10^6 and 11.3×10^6 CD34+ γ C + cells/kg body weight gene modified cells respectively [5]. Thus in each of these patients many proviral integrants exist with the potential to dysregulate many nearby genes, including proto-oncogenes.

5. The SCID-X1 trials as an example of genotoxicity in HSC gene therapy

SCID-X1 is a fatal X-linked inherited mutation of the IL2RG locus harboring the gamma c (γ C) cytokine receptor common subunit [5]. Inactivating mutations in this gene prevent proper cellular communication and maturation of lymphoid progenitor cells. The loss of cellular signaling in lymphoid progenitors prevents the development of mature T, NK, and B cells. Many SCID-X1 patients fail to thrive or suffer morbidity and mortality in early life because of impaired immune function, which leaves them susceptible to life-threatening infection. Allogeneic HSC transplantation has been used to treat SCID-X1, but many patients do not have suitable donors. In addition, graft versus host disease is a major source of mortality for patients treated by this approach. Graft versus host disease occurs when transplanted (allogeneic) immune cells from a donor recognize the host recipient tissue as foreign and attack these cells. SCID-X1 HSC gene therapy using the γ C transgene has a lower mortality rate and a higher treatment efficacy compared to conventional allogeneic bone marrow transplants [25], and is currently the only therapeutic choice for patients without a suitable donor. Prior to the French study, preclinical studies conducted in both murine and canine models corrected SCID-X1 deficiency with no reported adverse events [20, 31, 33, and 34].

In the French SCID-X1 trial, four of the ten patients developed T-cell leukemia from insertional mutagenesis from the murine moloney leukemia virus (MLV)-based vector [5, 25]. Careful molecular analysis of leukemic cells showed that the MLV provirus integrated near the proto-oncogene, LMO2, and suggested that viral enhancer elements in the provirus contributed to leukemia (Figure 2B) [25]. The SCID-X1 trials were the first HSC gene therapy clinical trial where vector-mediated insertional mutagenesis led to cancer. In this trial, MLV vector LTR enhancers activated LMO2 expression, resulting in T-cell LMO2 dependent proliferation (Figure 2B). LMO2 is normally silenced in mature T-cells, and when viral enhancers turn on expression, LMO2 drives T-cell proliferation by dysregulating transcription networks that affect the cell cycle. This promotes cell cycle escape and can result in higher proliferation rates compared to normal cells [35]. Leukemic transformation was a result of provirus integration near LMO2 with additional proviral integrants near other proto-oncogenes that resulted in expansion of cells with these mutations [5, 19].

6. Clonal expansion in the SCID-X1 trials

As evidenced in the French SCID-X1 trial, retroviral vector integration can dysregulate nearby host genes, thus affecting cell growth and survival. Once integrated, elements in the provirus, particularly enhancers in the LTR, can dysregulate nearby gene expression through several mechanisms (Figure 2). Aberrant gene expression can dysregulate host cell genes and regulatory networks involved with cell growth, including proto-oncogenes. A survival advantage can occur through the activation of proto-oncogenes giving cells a proliferative advantage or “go signal”. Alternatively, tumor suppressors can be inactivated, causing a proliferative advantage with uncontrolled cellular division from the lack of a “stop signal”. To date the genotoxicity described in HSC gene therapy trials has been through activation of growth promoting genes and proto-oncogenes, rather than inactivation of tumor suppressors. This is because activation requires a single integration into only one allele whereas inactivation of a tumor suppressor requires a second event, either a second integration or a loss of heterozygosity at that allele, to inactivate tumor suppressor activity. Tumor suppressors have however been identified in preclinical mouse studies [36-37]. These vector mediated mutations along with additional accumulating mutagenic events in expanding gene-modified repopulating cells can ultimately result in tumorigenesis. The proviral promoter and enhancer elements have been shown to act up to a distance of 500 Kb upstream and downstream of the site of proviral integration [38]. In the SCID-X1 trials where patients developed T-cell leukemia, the integration sites of dominant repopulating clones near the genes LMO2, BMI1, HMGA2, SEPT9, RUNX2, and RUNX3 gave rise to cells with a proliferative advantage or survival advantage over competitor repopulating cells. These advantages eventually led to an over-representation of these clones (Figure 3) [5].

We now know how vector-mediated dysregulation of these different genes may have contributed to clonal expansion and frank leukemia. LMO2 or Lim only 2 is a proto-oncogene that regulates early progenitor expansion during hematopoiesis [39]. LMO2 oncogenic properties were first observed in mature gene-modified T-cells, where it is not normally ex-

pressed [39]. However, after proviral integration events, resulting in activation of LMO2, these gene-modified T-cells begin to expand from dysregulation of transcriptional regulatory pathways. In 2010, Oram et al. demonstrated that LMO2 expression in T cells activates FLI1 and ERG enhancers, known to be involved in blood stem/progenitor cells. These gene products of FLI1 and ERG in turn activate the enhancer of the HHEX/PRH gene locus, which has been shown to act in early progenitor cell expansion and formation of T-lineage acute lymphoblastic leukemia (T-ALL) [35]. LMO2 overexpression has also been demonstrated to reduce or eliminate cell cyclin dependent kinase (CDK) inhibitors promoting escape of the G1 cell cycle checkpoints during cellular division [40]. Once aberrantly expressed within a cell, LMO2 promotes cell cycle progression via multiple mechanisms, giving the cell a proliferative advantage.

Additional proto-oncogenes were activated in the French SCID-X1 patients. The BMI1 proto-oncogene normally functions in self-renewal and maintenance of hematopoietic primitive stem cells [40, 41]. Like LMO2, dysregulation of BMI-1 via enhancer activation results in clonal expansion. Additional mechanisms mediate clonal expansion, such as the high mobility group AT-hook2 (HMGA2) which can provide a proliferative advantage when the endogenous gene is truncated via insertional mutagenesis. Truncation results in the loss of regulatory target sequences within the protein mRNA preventing degradation by the endogenous miRNA let7. These miRNA elements normally function to regulate HMGA2 via RNA interference using the RNA induced silencing complex (RISC) machinery [42]. Truncated HMGA2 mRNA is not degraded thus continuing activation of gene networks involved with cell proliferation, and cell-cycle progression [42]. Another mechanism that can contribute to clonal expansion is the aberrant expression of septin proteins. SEPT9 functions as a microtubule regulator and plays an important function in cytokinesis and chromosome segregation, thus affecting genomic stability [43, 44]. When aberrantly expressed, SEPT9 causes dysregulated cytokinesis or cell division resulting in missegregation of chromosomes [43, 44]. Genomic instability then results from SEPT9 dysregulation, leading to the accumulation of chromosomal deletions or amplifications from missegregation of chromosomes. These events in conjunction with additional mutations can enhance cell proliferation and survival.

Two additional genes identified near vector proviruses in the SCID-X1 trial were RUNX family members RUNX2 and RUNX3. RUNX proteins (RUNX 1, 2, 3) are a family of RUNT homology domain containing α -subunits that form heterodimeric transcription factors that mediate hematopoietic differentiation and expansion in conjunction with β subunit core binding factor (CBF). Aberrant expression of the RUNX proteins in mouse models hinders myeloid class progenitor differentiation capacity and represses expression of several target genes including Csf1R, Mpo, Cebpd, and the cell cycle inhibitor Cdkn1a [45]. Repression of these genes blocks hematopoietic stem cell differentiation leading to an accumulation of undifferentiated cells. These cells cannot pass the differentiation block to repopulate the depleted blood cell niche. The lack of differentiated mature cells continues to generate proliferative signaling pathways that further stimulate mutant HSC expansion. The expanded undifferentiated blast cells accumulate in the bone marrow, disrupting normal blood cell production, and can eventually give rise to various cytopenias and leukemic blast crisis.

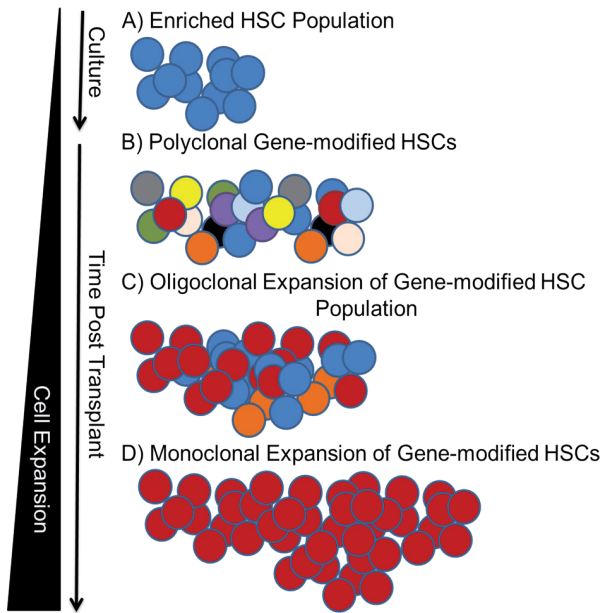


Figure 3. Clonal Expansion. A) Patient derived CD34⁺-enriched hematopoietic stem cells (HSC) population prior to ex vivo vector exposure. Untransduced cells are blue. B) Polyclonal proviral integration distribution in vector treated HSCs. This is an ideal proviral distribution, which HSC gene therapy would ideally maintain after infusion into patients. However, due to genotoxicity and selection pressures in vivo C) Oligoclonal expansion can be observed, where some clones expand. In some cases, D) individual clones may harbor a proviral integration near genes promoting a proliferative or survival advantage, which may eventually contribute to malignancy.

In summary, our current understanding of genotoxicity is that vector proviruses dysregulate the expression of key regulators of cell cycle, cell survival, genomic stability, proliferative gene networking and cellular differentiation. This leads to an over-representation of gene-modified clones with these mutations in the peripheral blood (PB) and bone marrow (BM) which is referred to as clonal expansion (Figure 3). Clonal expansion can lead to additional mutations that can eventually cause frank leukemia. The leukemias observed in the French SCID-X1 trial refocused the gene therapy field to better understand the mechanisms of vector dysregulation of host genes, with the ultimate goal of reducing the risk of genotoxicity.

7. Risk factors for clonal expansion

It is important to remember that the SCID-X1 trials are one example of clonal expansion in a specific disease setting using a specific vector type with a specific transgene. Several factors can influence clonal expansion including the type of vector, vector design, the therapeutic transgene and the disease setting. For example the transgene in and of itself may provide a

selective advantage to cells through its expression. This was true in the SCID-X1 trials, where expression of the corrective γ C transgene gave cells a proliferative advantage allowing reconstitution of the lymphoid cell population from a modest number of gene modified HSCs [5]. To determine the mechanisms behind these expansion events investigators must first characterize the integration sites of the vectors being used to deliver the therapeutic transgene. This allows identification of nearby genes that may have been dysregulated leading to clonal expansion.

8. Vector integration sites in expanded repopulating clones allow clonal tracking

To understand the risk of genotoxicity we need to identify where different vectors tend to integrate. Conveniently, integrated vector proviruses serve as molecular tags to identify integration sites and to track specific clones in order to study clonal expansion. Sequencing the unique vector-chromosome junctions can identify where in the genome the virus has integrated. Analysis of vector insertion sites has allowed researchers to compile comprehensive integration profiles for specific virus types and assess the safety of viral vectors based on the regions of preferred or recurrent integration.

Long term tracking of gene-modified cells is necessary to monitor potential adverse events that may occur over time resulting in clonal expansion. By identifying the spectrum of vector integration sites in repopulating cells, the clonality of repopulating cells can be estimated and the expansion of specific clones can be monitored. Long-term tracking may also provide insight into specific mechanism of clonal expansion, such as emergent LMO2 expansion in SCID-X1 trials, and will direct novel approaches to reduce genotoxic effects [46]. It has become an important area of study to understand where retroviruses integrate in the human genome, thus affecting their safety for use in HSC gene therapy approaches.

9. The integration profile of retroviruses and its relation to genotoxicity

Identifying the integration profiles of different vector types has provided important data on the relative genotoxic risk associated with different vectors. Following the leukemias observed in the SCID-X1 trial, integration site distributions were described for different retroviral vectors being developed for gene therapy. Viral integration sites for HIV-1, MLV and foamy virus (FV) vectors were reported and each exhibits a specific and unique integration profile. HIV-1 based vectors showed preferences for integration within actively transcribed genes [47] whereas MLV vectors tends to integrate within transcription start sites near CpG islands [48-50]. FV vectors also preferentially integrate near transcription start sites and CpG islands but less frequently than MLV vectors and integrate less frequently in genes than HIV vectors. The propensity of MLV-based vectors to integrate preferentially very close to promoter regions was of significant concern since this may increase the risk of dysregulating

proto-oncogenes. The integration profiles were found to be largely independent of the route of entry [47, 51, 52] and target cell type [49, 53, 54] although characteristics such as cell cycle of the target cell can play a minor role in the profile [49,54].

The factors that contribute to the integration profile of viruses and viral vectors are greatly influenced by a complement of host proteins that interact with a poorly defined retroviral pre-integration complex (PIC). The PIC is a complex of proteins associated with the viral genome, and during infection, the PIC must migrate to the nucleus to mediate integration of the reverse-transcribed viral DNA to generate the vector provirus. This process and the associated proteins that affect it have been studied using various methods [55-57]. Viral Gag and integrase proteins have been shown to interact with chromatin, affectively tethering the PIC to specific chromosomal regions, thus directing integration [57, 58]. Studies have compared the contributions of the viral integrase and Gag proteins using MLV-HIV chimeras, and shown that both play important roles in integration site specificity [57]. The HIV lens-epithelium-derived growth factor (LEDGF) is a host derived tethering protein that has been demonstrated to associate with the PIC and chromatin affecting HIV-1 integration patterns. This host protein has a strong binding affinity for HIV integrase proteins, which are associated with the lentiviral PIC [59]. The tethering of the PIC to LEDGF protects the PIC from host enzymatic defenses [56], promotes chromatin binding [57, 60], and directs integration site distribution [61]. Unique to foamy virus biology, the c-terminal end of the Gag protein contains glycine-arginine motifs known as a GR boxes [62]. These boxes direct viral packaging [63, 64] and nuclear localization [62, 64]. In addition to these features, a 13 amino acid motif called the chromatin-binding site (CBS) has been characterized [58]. This CBS contains a functional binding domain for core histones H2A/H2B that is thought to tether the PIC to the chromatin after translocation into the nucleus [58]. Host chromatin tethering proteins often associate with the PIC complex and affect integration site distributions. Better characterization of cell-virus interaction should enhance our understanding of viral integration patterns. This has potentially led to novel approaches to direct vector integrations to "safe harbor" chromosomal regions, that do not have genes that can lead to clonal expansion when dysregulated.

10. Methods for integration site analysis

Many methods exist for generating retroviral insertion site data. PCR based techniques include ligation mediated PCR (LM-PCR Figure 4A), Linear amplification-mediated PCR (LAM-PCR Figure 4B), and non-restrictive LAM-PCR (nrLAM-PCR Figure 4C). LM-PCR relies on frequently cutting restriction enzymes to generate fragments that contain the provirus: chromosome junction. These fragments are then ligated to linkers, and after several rounds of PCR, the resulting products are sequenced. LAM-PCR uses an LTR-specific primer in several rounds of 'linear' amplification where the LTR: chromosome junction is amplified. Nested PCR is then used to produce products that can be directly sequenced or transformed into bacteria and sequenced. nrLAM-PCR is similar to LAM-PCR but uses random shearing rather than digestion of DNA with restriction enzymes prior to linker ligation and sequencing, thus avoiding restriction site bias and is currently the gold standard in the field.

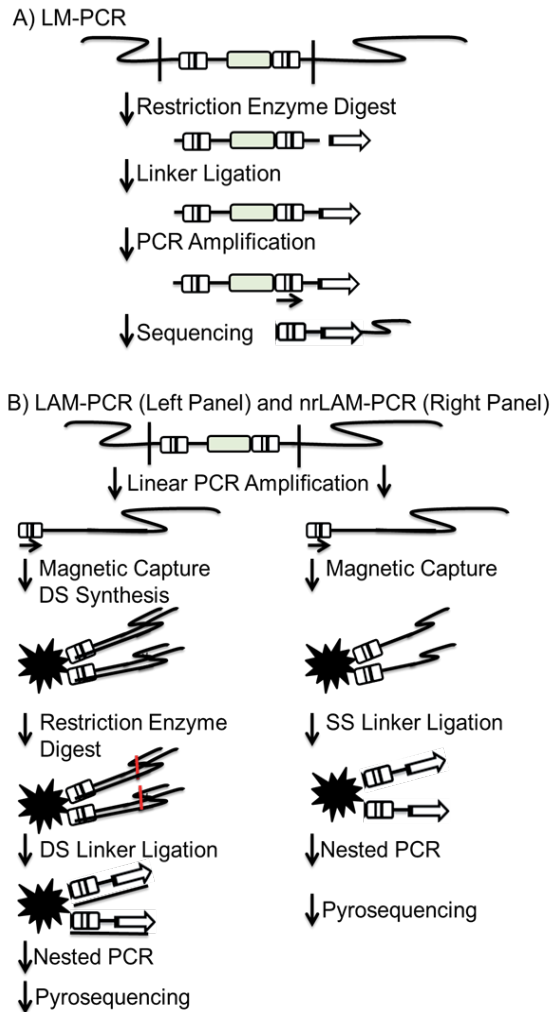


Figure 4. PCR Based LTR: Chromosome Junction Sequencing. A) Demonstration of ligation-mediated PCR, where genomic DNA is cut by restriction enzyme digestion, ligated to a linker, and amplified before sequencing of oligos with an LTR specific primer. B) Left Panel: Linear-amplification-mediated PCR (LAM-PCR) amplifies regions of genomic DNA containing integrated vector proviruses using an LTR specific primer. The resulting oligos are captured on magnetic beads and double strand synthesis is performed, followed by restriction enzyme digestion and ligation of a double stranded linker. Nested PCR is then used and the resulting products sequenced. Right Panel: Non-restrictive linear-amplification-mediated PCR (nrLAM-PCR) amplifies genomic DNA with integrated vector proviruses with an LTR specific primer. The resulting products are enriched on magnetic beads, followed by single strand linker ligation. Nested PCR is then employed and the products are sequenced.

One limitation of the above methods is that PCR bias can affect the frequency of detected integration sites [65, 66]. Another method that has been used is shuttle vector rescue technology, which eliminates PCR-based bias [67, 68, and 54]. In shuttle vector rescue, vector plasmids encode a bacterial origin of replication and selection gene. DNA fragments that contain the shuttle vector LTR: chromosome junction are ligated and then transformed into bacteria. These bacteria can then be grown as colonies to amplify plasmid clones of each potential insertion site in the absence of PCR based skewing (Figure 5). Plasmid DNA is then extracted from bacterial colonies and sequenced with an LTR specific primer. In all of the above methods, aligning the genomic sequence immediately next to the proviral LTR to a published genome databases allows for identification of the proviral integration site. It will be interesting to compare the shuttle vector approach to nrLAM-PCR in animal models to provide information on any potential bias from either technique.

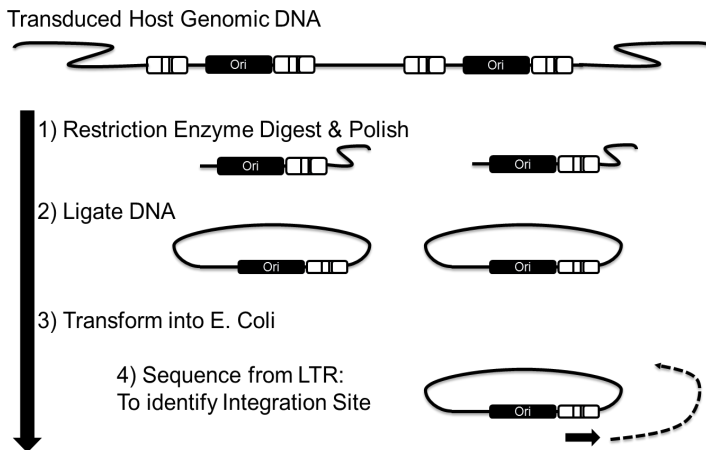


Figure 5. Plasmid Shuttle Vector Rescue. Genomic DNA is presented with vector integrations showing the proviral 5' LTR, the genetic element encoding a bacterial origin of replications (Ori), and the vector provirus 3' LTR. Vector exposed cells are lysed and genomic DNA harboring integrated vector proviruses collected. The genomic DNA harboring proviral integrants is fragmented by restriction enzyme digest, and then self-ligated to form plasmids which may contain portions of the provirus encoding a bacterial origin of replication and an antibiotic selection gene. These plasmids are then transformed into E. coli. E. coli transformed with plasmids containing the bacterial origin and antibiotic resistance gene will form colonies. Sequencing the colony plasmids identifies proviral LTR: chromosome junctions.

11. Animal models to study genotoxicity: Tumor prone mouse models

Animal models allow the *in vivo* study of the genotoxicity of HSC gene therapy approaches, within specific disease contexts. These studies are critical because while *in vitro* genotoxicity assays can provide important information on the relative genotoxicity of different vectors [50], only animal models can assess genotoxic effects on *in vivo* hematopoiesis. Tumor prone mouse

models have provided important data on the relative genotoxicity of different vectors systems and have identified genes and gene networks involved in vector-mediated malignant transformation [69]. The advantage of tumor prone mice is that the frequency of clonal expansion and tumorigenesis resulting from vector-mediated genotoxic events is increased, thereby allowing readout of vector-mediated malignancy within the life span of a mouse.

Several studies have focused on gammaretroviral and lentiviral vectors; testing vectors with hybrid LTRs from both viral systems to identify the elements responsible for different genotoxicities. These studies, in conjunction with tumor-prone mouse models, have informed vector design modifications. Enhancer deletion, use of internal housekeeping promoters, and deletions of vector cryptic splice sites can be used to reduce genotoxic events and improve safety [69]. The tumor prone *cdkn2a*^{-/-} mouse model has been used to compare retroviral insertional oncogenic potential using MLV and HIV-based vectors in *in vivo* genotoxicity assays [70]. These assays demonstrated HIV- based lentiviral vectors exhibited an improved safety profile compared to MLV based vectors. The *Cdkn2a* locus controls cell senescence and has been shown to prevent cell transformation. Inactivation of this gene promotes malignancy and has been implicated in almost all types of human cancer [71, 72]. These studies have compared the genotoxic contribution of vector components such as strong LTR promoters. They have also shown the ability of self-inactivating LTR designs to reduce genotoxicity.

Although these studies primarily identify activated proto-oncogenes, it is also possible to identify dysregulated tumor suppressors using retroviral mutagenesis screens [73-75]. Proviruses can downregulate nearby host gene transcription via host cell methylation of the proviral LTR that also leads to methylation of the nearby host genes. Identification of the vector provirus location and nearby host genes can be used to identify haplo-insufficiencies related to malignancy. Recent observations of viral LTR methylation causing proviral transgene silencing can now be used to identify down regulation of host genes near the methylated proviral integration sites [75]. The vector LTR methylation events and subsequent silencing of host genes can identify potential tumor suppressors related to vector-mediated genotoxicity. A recent study used methylation specific PCR and methylated DNA immunoprecipitation assays to analyze methylated proviral integrations in mutagenized mouse tumors [75]. In this study the identification of the methylated and downregulated gene *PTP4A3* in MLV vector-mutagenized murine leukemia samples, suggests that haplo-insufficiency may be involved in retroviral genotoxicity [75]. This study also suggests that future studies may identify vector-mediated haploinsufficiency genes that contribute to genotoxicity.

12. Large animal models of genotoxicity

Large animal models allow long term monitoring of HSC genotoxicity due to the longer life span of large animals such as dogs and nonhuman primates relative to mice. These models are important to assess the long-term risks associated with malignant transformation follow-

ing insertional mutagenesis from clonal expansion and long-term selection pressures. In two non-human primate studies, the distribution of MLV-based gammaretroviral and SIV and HIV-1 based lentiviral integration sites were evaluated over long periods [76, 77]. Clonal expansion or malignant transformation was not observed. However, integrants were observed at higher than expected frequencies near growth promoting genes and proto-oncogenes. This suggests that repopulating cells with integrations near these genes can influence survival of those clones. These studies can shed light on potential mechanisms of clonal expansion and can allow comparison of different vector types. However, these studies in normal animals using vectors with a reporter gene that is not expected to provide a selective growth advantage did not predict the clonal expansion observed in the SCID-X1 trial. This suggests that large animal models of specific disease settings such as the SCID-X1 dog model [34] will be important to test improved vectors designed to reduce genotoxicity in a specific disease setting.

Another important contribution of large animal models has been to improve our understanding of the effects of ex vivo culture on clonal expansion. It has been shown in a nonhuman primate model that genotoxicity can be significantly influenced by the culturing conditions of gene-modified cells ex vivo. In this study, only six days of culture increased the incidence of specific clones with gamma retroviral vector integrations near MDS/ EVI1 locus associated with a leukemic phenotype [78]. To monitor the potential effects of ex vivo culturing condition and in vivo selection on gene-modified repopulating cells, clonal tracking methods must be employed.

13. Tracking of genetically modified clones

The above studies have identified mechanisms of genotoxicity and clonal expansion. During clinical trials, it is important to monitor potential clonal expansion in order to understand genotoxicity and to anticipate potential adverse events [79]. As an example, dysregulation of HMGA2 in a clinical trial for β -thalassemia resulted in clonal expansion of gene-modified cells that has provided a therapeutic benefit without malignancy to date [80]. β -thalassemia is a genetic deficiency that hinders β -globin production and patients with this mutation are reliant upon continued blood transfusions to restore normal blood globin levels. Before HSC gene therapy the only therapeutic available was allogeneic transplant, however the procedure is high risk and patient limited due to a lack of matched donors. Thus, patients risk transplant rejection or development of graft versus host disease. To achieve therapeutic benefit using HSC gene therapy, lineage specific transgene expression in erythrocytes is required, promoting appropriate β -globin expression. Therapeutic benefit was achieved in two gene therapy patients resulting from a partially dominant clone harboring proviral insertions near HMGA2 [80]. The authors of this study conclude the clone with HMGA2 may remain homeostatic or eventually progress through multistep leukemogenesis, indicating a

strong need for continued gene-marking studies and clonal tracking of these gene-modified cells in vivo [80].

14. MDS1/EVI1, PRDM16, SETBP1 in trial for CGD

Chronic Granulomatous disease (CGD) is an x-linked inherited immunodeficiency resulting from a mutation in one of the NADPH oxidase genes [87]. The gp91^{phox} protein accounts for 70% of cases [81]. The gp91^{phox} transgene has been used in corrective HSC gene therapy clinical trials [81]. Unlike SCID-X1 gene-modified cells, CGD gene-modified cells do not exhibit a proliferative advantage from transgene expression. The lack of conditioning or selection of gene-modified cells contributed to a loss of therapeutic benefit and detection of gene-modified cells. Patients in the initial trials had low marking with gene expression of the corrected transgene for short periods of therapeutic benefit. Adverse genotoxic events developed 2 ½ years after the initial therapy, with clonal expansion and leukemic transformation [7, 82]. Clonal analysis found activation of MDS1/EVI1, PRDM16, and SETBP1 proto-oncogenes [81]. One of the patients died in treatment from complications arising from the leukemia, the other patient survived after receiving an additional allogeneic transplant. In this study there was inefficient engraftment and short-term transgene expression, with vector silencing via vector LTR methylation (Figure 2D) [83]. Further improvements to enhance engraftment and selection of gene-modified cells after infusion are needed for HSC gene therapy to treat CGD.

15. CCND2 and MDS1/EVI1 trial for Wiskott-Aldrich

HSC gene therapy has also been used in the treatment of Wiskott-Aldrich syndrome (WAS), an X-linked recessive immune disorder. In this study, patients underwent conditioning with busulfan to enhance the engraftment of gene-modified cells [84]. Patients exhibited therapeutic benefit, with resolution of disease symptoms, although clonal skewing was detected for clones that harbored vector integration sites near CCND2 and MDS1/EVI1 [84]. Despite the high success of WAS HSC gene therapy in nine of the ten patients treated, patient 2 was reported to have experienced vector-derived genotoxic events after more than 3 years of therapeutic benefit [84], ultimately resulting in T-cell leukemia. The leukemia was a result of proviral integration near the gene LMO2, and this patient has since been treated with chemotherapy resulting in remission [85-87].

Clonal tracking in vivo has recently been employed in a study where patients with glioblastoma were given gene-modified hematopoietic repopulating stem cells carrying a methylguanine methyltransferase mutant (MGMT-P140K) [17]. In this approach gene-modified hematopoietic repopulating cells expressing this mutant enzyme are resistant to O6-benzylguanine (O6BG). This allows treatment of the glioblastoma solid tumor with O6BG and an alkylating agent. By protecting the hematopoietic system from chemotherapy-

mediated hematopoietic toxicity, higher doses of chemotherapy can be used to treat the glioblastoma. In patients undergoing chemotherapy, gene-modified cells were monitored to track potential clonal expansion and to assess patient safety. Repopulating cells were tracked and their retroviral integration sites monitored at several different time points, pre- and post-chemotherapeutic treatment. Throughout the course of chemotherapy treatment, over 12,000 unique retroviral insertion sites (RISs) were present in the three treated patients. The heterogeneity of RISs suggests a highly polyclonal engraftment of gene-modified repopulating cells. During tracking two patients exhibited clonal expansion, with prominent clones appearing with vector proviruses in PRDM16 (PR domain-containing 16), Set binding protein 1 (SETBP1), and high-mobility group A2 (HMGA2) genes.

In summary, it is clear that HSC gene therapy is an efficacious therapeutic approach, able to treat debilitating and often fatal genetic deficiencies. However, the observed clonal expansion in these early clinical trials presents a major concern in the field. There is a need for vectors with an improved safety profile that are less likely to dysregulate genes and lead to clonal expansion.

16. Next-generation vectors: Reducing genotoxicity

Extensive efforts are underway to develop vector systems with safer integration profiles and reduced genotoxic effect. One approach is to retarget vector integration using tethering proteins that redirect the PIC. Other efforts focus on reducing genotoxicity by producing vectors less likely to dysregulate nearby genes. Such vectors include self-inactivating LTRs, which have deleted enhancer elements or U3 regions, preventing enhancer mediated expression of nearby genes. Newer vectors are also able to regulate context dependent transgene expression using insulators and repressor elements to prevent viral promoters from activating genes near the site of insertion [88]. Recently investigators have also identified insertion-al effects mediating alternative splicing, producing aberrant splice variants and protein fusion products causing oncogenesis [89, 90]. Modifying the vector-borne cryptic splice sites in vector backbones can create safer vectors reducing aberrant splice variant, reducing post-translational dysregulation of gene expression (Figure 2 C), [89, 91-93]. In addition, vector and host miRNAs have recently been explored. An example of miRNA control was demonstrated using miRNA let7 control elements, regulating expression of transgenes in stem cells versus somatic cells. Silencing of the transgenes occurs in somatic mature cells by miRNA cleavage sites. When let7 target sequence is matured and expressed, cleavage of the transgene containing the target sequence occurs [94]. In pluripotent cells, let7 is not expressed, thus the target sequences are not cleaved and full-length transgene is expressed [94]. This technology could potentially direct HSC gene therapy over a major hurdle, by reducing vector-born genotoxicity through transgene expression in a highly controlled, cell specific context. These miRNA technologies have the ability to restrict transgene expression to a specific cell type and are even able to restrict transgene expression within a specific differentiation stage of that cell type, allowing a more specific control of transgene delivery, dosage, and

expression [95]. Incorporation of miRNA technologies can improve vector efficacy and safety, ultimately reducing or limiting vector-born genotoxic events.

17. Chromatin insulators

Chromatin insulators are being developed to reduce the propensity of integrated vector proviruses to dysregulate host gene expression. Insulators are DNA elements that repress the activity of enhancers on promoters. The chicken hypersensitive site-4 (cHS4) insulator contains five DNA binding elements within a 250 bp fragment known as the dominant DNase hypersensitive site [96, 97]. A 650 bp cHS4 element has been characterized in conjunction with a 400 bp element from cHS4 that can sufficiently block enhancer activation [98]. Additional insulators have been described for sea urchin *sns5* insulator and an adeno-associated (AAVS1) viral insulator DHS-S1 [99, 100].

The cHS4 insulator has been used in several retroviral vector systems [80,99, 101-106]. Initial studies with cHS4 lentiviral vectors were shown to be effective in reducing genotoxicity [107]. Their use in erythrocytes gave encouraging results, albeit with low titers. In addition, this study also demonstrated the effects of insulator failure after a reduction of cHS4 element repeats, which was reported to have contributed to insertional mutagenesis and expansion of clones harboring HMGA2 mutations [80]. Sea urchin *sns5* is a 462 bp insulator region that was demonstrated to function in gamma retroviral vectors by maintaining chromatin position affects [100]. This element also contains a previously identified insulator region of 265 bp found to block enhancer-activated directional transcription in human cells [108, 109]. The DHS-S1 viral insulator has been demonstrated to increase transgene expression 1000-fold from an elongation factor 1-alpha (EF1 α) promoter in muscle cells, but was not studied for its ability to block transactivation of host genes [99]. Insulators can potentially serve several major functions, by protecting against vector silencing, moderating vector variegation or uniformity of expression, and protecting nearby host genes from enhancer activation. Additional studies should help better characterize the efficacy of insulated vectors

18. Incorporation of cell-type specific control elements

Incorporation of cell-type specific control elements such as erythrocyte specific enhancer-promoter has been used to control transgene expression [110]. The use of a lineage specific promoter ensures that transgene expression only occurs within the lineage from which the promoter is active. Moreover, avoiding expression of the transgene in other cell types with which the promoter is not active. The premise of lineage-restricted promoters for HSC gene therapy is that they may eliminate or reduce genotoxicity resulting from dysregulation of genes in stem/progenitor cells. This is accomplished by activating transgene expression only in a cell lineage with which transgene expression is required for therapeutic benefit. This ap-

proach might protect primitive cells from dysregulation, as the promoter is not expressed until differentiation into the target cell type. This is an attractive area of research for diseases that characteristically are exhibited in one lineage of the blood system such as hemaglobinopathies. This approach was used in a thalassemia trial, where a β -locus-control-region-derived promoter was used [80]. The transgene is delivered to long term repopulating HSCs, but the promoter is not active. Only after erythroid differentiation would the enhancer become active, resulting in transgene expression in erythrocytes. This may reduce the occurrence of proto-oncogene activation in stem/progenitor cells. Other lineage specific promoters are being studied, including B cell lineage specific promoters [111]. When a lineage specific promoter is not a viable option, vectors may need to be targeted to specific regions of the chromatin, where vector insertion is at a much lower risk of causing malignancy.

19. Re-targeting of retroviral vectors

Efforts have been made to target retroviral proviruses to specific chromosomal locations. LEDGF, a host cell protein that interacts with HIV Gag has been used to effect tethering and targeting of viral integration Gijsbers et al [112]. In this study, cells were modified to express LEDGF protein containing a chromatin-interacting domain of chromobox homolog 1 (CBX1), which binds di- and tri- methylated regions of histone 3 (H3) in heterochromatic regions of the genome [112]. H3s are located pericentric to regions of heterochromatin, which is safer in terms of insertional mutagenesis as genes in these regions are normally silent. However, the reporting of significant re-targeting of integration sites to heterochromatin is encouraging.

In addition, authors reported that transgene expression was not affected by targeting to these transcriptionally unfavorable heterochromatic sites [112]. These exciting experiments have demonstrated that vectors containing Gag and Pol C termini with adapted or unique binding domains could direct insertional distribution [113]. However, LEDGF cannot be modified in HSC gene therapy and alternative tethering approaches must be devised.

Additional tethering proteins have been studied and need to be fully characterized to expand targeted integration locations in in vivo approaches [113]. In future studies use of appropriate tethers for modified integration site preference may reduce genotoxicity and may provide a better understanding of virus and host interactions affecting viral integration. In addition, even with vector systems that have incorporated these safety mechanisms, genotoxic events may arise and methods to ablate the gene-modified cells will be useful to avoid malignancy.

20. Approaches to ablate expanded cell clones

Several approaches exist to ablate or control expanded clones after insertionally activated oncogenesis has occurred. Conditional selection systems have been employed to control the

longevity and survival of HSC gene-modified clones after infusion. Several conditional promoter systems such as TET on/off and pro-drug inducible expression cassettes have been used to target cancer cells harboring dangerous integrations through vector silencing and suicide gene activation [114].

The tetracycline (Tet) on/off gene expression system utilizes a pro-drug to regulate transgene expression by modifying a Tet repressor protein (TetR). TetR is constitutively expressed and depending on its conformation will either be bound to the tetracycline operator (TetO), or unbound. In a Tet on system, TetR does not bind TetO until administration of the pro-drug, typically doxycycline. Once the pro-drug is administered, TetR actively binds TetO and silences transcription of nearby transgenes. In the absence of the pro-drug TetR cannot bind TetO, and this region of the genome is no longer blocked from transcription, and gene expression resumes. Alternatively, modifications have been made to TetR, allowing it to bind to repressor sequences until it is deactivated by a pro-drug; this system is called Tet off. The Tet on/off system may be used in conjunction with suicide genes to ablate undesired clones.

In gene suicide approaches, HSC gene therapy delivers an active transgene in conjunction with a pro-drug induced suicide gene such as Thymidine Kinase [115]. In the event that transformations result from insertional mutagenesis and clonal expansion, clones harboring integrations can be eliminated or reduced by activating expression of the suicide gene, inducing apoptosis and eliminating clones harboring proviral integrations [26]. Recent clinical trials using an inducible caspase 9 (iCasp9), which remains in an inactivated state until dimerization following treatment with AP1903 small molecule, was reported in four patients with graft versus host disease after gene-modified hematopoietic transfusion [116]. In four patients, a single infusion of AP1903 was reported to have eliminated 90% of gene modified T cells within 30 minutes of administration of the inducing drug AP1903. GVHD and other associated illnesses typically observed after allogeneic bone marrow transplants were not detected up to a year after AP1903 treatment [116]. Thymidine kinase and iCasp9 present effective safety switches to control an array of genotoxic effects arising from HSC gene therapy [26, 117]. Utilizing these safety mechanisms in new vector designs will aid in furthering safety and reducing genotoxic events, and allow for selective ablation of expanded gene-modified clones in vivo.

21. Concluding summary

The use of HSC gene therapy in clinical trials is expanding, and the therapeutic potential is enormous. Following the initial successes with ADA SCID and SCID-X1 additional efficacious therapies were reported for WAS, β -thalassemia, and CGD. Seymour et al. reported that the majority of over 90 patients receiving HSC gene therapy exhibit prolonged clinical benefit, with greater than 90% survival rate despite the occurrence of genotoxic events [46]. Current studies that are underway aim to characterize and reduce genotoxicity. Several approaches have reduced genotoxic events in preclinical studies. With ongoing technological

refinement, newer and safer HSC gene therapy vectors are entering, or will soon enter, the clinical arena. These advances are crucial for HSC gene therapy to enter mainstream medicine as an effective and safe therapeutic approach.

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Efficient AAV Vector Production System: Towards Gene Therapy For Duchenne Muscular Dystrophy

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Choice of vector

Successful gene therapy requires an adequate level of long-term transgene expression in the target tissues. While various viral vectors have been considered for the delivery of genes *in vivo*, an adeno-associated virus (AAV)-based vector is emerging as the gene transfer vehicle with the most potential for use in the neuromuscular gene therapies. The advantages of the AAV vector include the lack of disease associated with a wild-type virus, the ability to transduce non-dividing cells, and the long-term expression of the delivered transgenes.[1] Some serotypes of recombinant AAV (rAAV) exhibit a potent tropism for striated muscles.[2] Therefore, a supplementation of secretory protein can be achieved with this vector to use intramuscular injection.[3] Since a 5-kb genome is considered to be the upper limit for a single AAV virion, various truncated genes could be provided to meet size capacity, if necessary.[4]

Due to ingenious cloning and preparation techniques, adenovirus vectors are efficient delivery systems of episomal DNA into eukaryotic cell nuclei.[5] The utility of adenovirus vectors has been increased by capsid modifications that alter tropism, and by the generation of hybrid vectors that promote chromosomal insertion.[6] Also, gutted adenovirus vectors devoid of all adenoviral genes allow for the insertion of large transgenes, and trigger fewer cytotoxic and immunogenic effects than do those only deleted in the E1 regions of the adenovirus early genes.[7] Human artificial chromosomes (HACs) have the capacity to deliver genes in any size into host cells without integrating the gene into the host genome, thereby preventing the possibility of insertional mutagenesis and genomic instability.[8]

Long-term correction of genetic diseases requires permanent integration of therapeutic genes into chromosomes of the affected cells. However, retrovirus vector integration can trigger deregulated premalignant cell proliferation with unexpected frequency, most likely driven by retrovirus enhancer activity on the LMO2 gene promoter. [9] A goal in clinical gene therapy is to develop gene transfer vehicles that can integrate exogenous therapeutic genes at specific chromosomal loci as a safe harbor, so that insertional oncogenesis is prevented. AAV can insert its genome into a specific locus, designated AAVS1, on chromosome 19 of the human genome.[10] The AAV Rep78/68 proteins and the Rep78/68-binding sequences are the trans- and cis-acting elements needed for this reaction. A dual high-capacity adenovirus-AAV hybrid vector with full-length human dystrophin-coding sequences flanked by AAV integration-enhancing elements was tested for targeted integration.[11]

1.2. AAV biology

AAV is a small (20-26nm) non-enveloped dependent parvovirus with a single-stranded linear genome that contains two open reading frames (*rep* and *cap*).[12] The viral genome is characterized by the inverted terminal repeats (ITRs) to flank these open reading frames (Figure 1A). The genome encodes four replication proteins (Rep78, Rep68, Rep52, and Rep40) and three capsid proteins (Cap: VP1, VP2, and VP3). The large Rep (Rep78 and Rep68) proteins regulate AAV gene expression and hold nicking activity at the terminal resolution site as well as binding activity at Rep binding elements to process AAV replication (Figure 1B). The small Rep proteins (Rep52 and Rep40) are used for the accumulation of single-stranded viral genome followed by packaging within AAV capsids.

The minimum sets of regions in helper adenovirus that mediate AAV vector replication are the E1, E2A, E4, and VA.[13] A human embryonic kidney cell line 293 encodes the E1 region of the Ad5 genome.[14] The helper plasmid assembling E2A, E4, and VA regions (Ad-helper plasmid) is cotransfected into the 293 cells, along with plasmids encoding the AAV vector genome (vector plasmid) as well as *rep* and *cap* genes (AAV-helper plasmid). AAV vector is produced as efficiently as when adenovirus infection is employed as a helper virus. Furthermore, contamination of most adenovirus proteins can be avoided in AAV vector stock made by this helper virus-free method.

1.3. Vector application using various serotypes

The preparation of AAV vector for gene therapy study of neuromuscular diseases is greatly facilitated. Although AAV2 has been the serotype most extensively studied in preclinical and clinical trials, recently we have focused on the use of AAV vectors pseudotyped with capsid protein of alternative serotypes. A number of primate AAV serotypes have been characterized in the literature and are designated. There is divergence in homology and tropism for various AAV serotypes. For instance, the homology with capsid protein is only about 60% between AAV2 and AAV5[15], therefore the capsid structure could be responsible for the improved transduction efficiency.

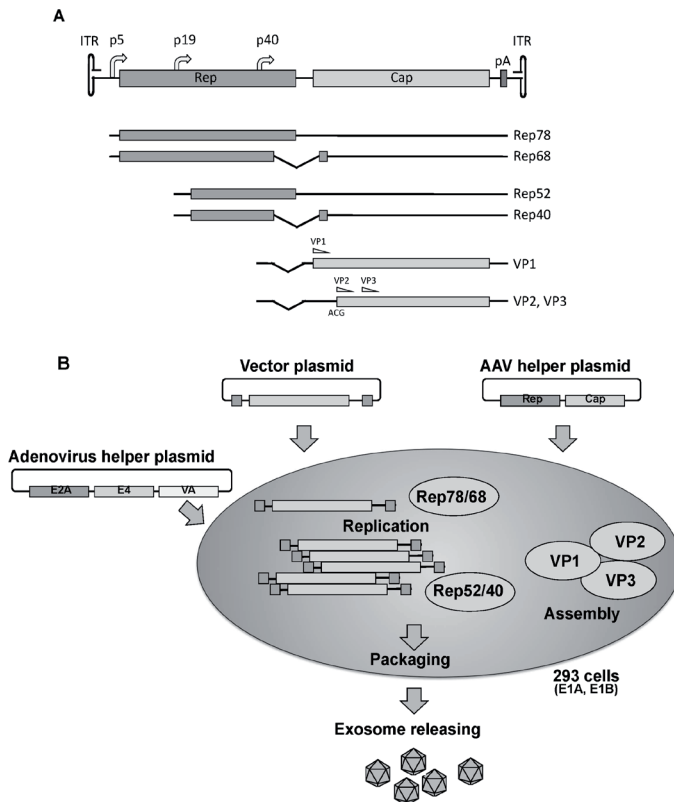


Figure 1. A) The *rep* and *cap* genes flanked by ITRs. The large Rep proteins (Rep78 and Rep68) are produced from transcripts using p5 promoter, while small Rep (Rep52 and Rep40) are produced from p19 promoter. (B) Recombinant AAV production. AAV has productive infection in the presence of adenovirus helper regions (E1, E2A, E4, and VA). This process is characterized by genome replication, assembly of the capsid proteins (VP1, VP2, and VP3), and packaging leading to virion production along with exosome releasing.

We found that choice of AAV serotypes and promoters could be quite useful for targeted transgene expression. For instance, the transgene expression of rAAV5 with the Rous sarcoma virus (RSV) promoter was preferentially found in the granular cells of the gerbil hippocampus, whereas transgene expression of rAAV2 with the RSV promoter was found in the pyramidal and granular cells.[16] Since AAV3 vector can specifically transduce cochlear inner hair cells with high efficiency *in vivo*, rAAV-mediated transduction might be promising for gene replacement strategies to correct recessive genetic hearing loss due to monogenic mutation.[17] Also, there is a significant difference in transgene expression by various AAV serotypes transduced into muscle. We observed that intramuscular injection of AAV5-IL-10 promoted a much higher serum level of secreted transgene product, as compared to AAV2-

mediated transfer.[18] We further demonstrated that AAV1 could more efficiently transduce the muscle than AAV5. Intramuscular single injection of modest doses of rAAV1 expressing IL-10 (6×10^{10} g.c. per rat) introduced therapeutic levels of the transgene expression over the long-term to treat pulmonary arterial hypertension.[3] rAAV1-mediated sustained IL-10 expression also significantly ameliorated hypertensive organ damage to improve survival rate of Dahl salt-sensitive rats.[19] Furthermore, this protein supplementation therapy by rAAV1-mediated muscle transduction was quite effective to prevent vascular remodeling and end-organ damage in the stroke-prone spontaneously hypertensive rat.[20] Interestingly, alpha-sarcoglycan expression with single intramuscular injection of rAAV8 was widely distributed in the hind limb muscle as well as cardiac muscle, and persisted for 7 months with a reversal of the muscle pathology and improvement in the contractile force in the alpha-sarcoglycan-deficient mice.[21] Intravenous administration of rAAV8 into the hind limb in dogs resulted in improved transgene expression in the skeletal muscles lasting over a period of 8 weeks.[22] Moreover, rAAV9 would be administered systemically with excellent cardiac tropism.[23] Further strategies have been attempted to discover novel AAV capsid sequences from primate tissue, which can be used to develop newer-generation rAAVs with a greater diversity of tissue tropism for clinical gene therapy.

1.4. scAAV

Clinical gene therapy often requires rapid transduction with reasonable efficiency. In the case of AAV, second strand synthesis of the vector genome in the nucleus is the rate-limiting step for efficient transduction. Therefore, self-complementary AAV (scAAV) vector would be quite promising to promote efficient transduction regardless of DNA synthesis or annealing.[24] The scAAV vectors can bypass the inter-molecular annealing or second-strand synthesis by using intra-molecular annealing to immediately form transcriptionally active double-stranded DNA (Figure 2). Although immediate and efficient transduction could be observed with scAAV, the maximal insert size of the transgene cassette is reduced to 3.3 kb.[25]

2. Effective production strategies of rAAV

2.1. Principle of production

To gain acceptance as a medical treatment with a dose of over 1×10^{13} genome copies (g.c.)/kg body weight, therapeutic strategies with AAV vectors require a scalable and provident production method. However, the production and purification of recombinant virus stocks with conventional techniques entails cumbersome procedures not suited to the clinical setting. Therefore, development of effective large-scale culture and purification steps are required to meet end-product specifications.

A production protocol of AAV vectors in the absence of a helper virus[13] is widely employed for triple plasmid transduction of human embryonic kidney 293 cells.[1] The adenovirus regions that mediate AAV vector replication (namely, the VA, E2A and E4 regions) were assembled into a helper plasmid. When this helper plasmid is co-transfected into 293

cells along with plasmids encoding the AAV vector genome and *rep-cap* genes, the AAV vector is produced as efficiently as when using adenovirus infection. Importantly, contamination of most adenovirus proteins can be avoided in AAV vector stock made by this helper virus-free method.

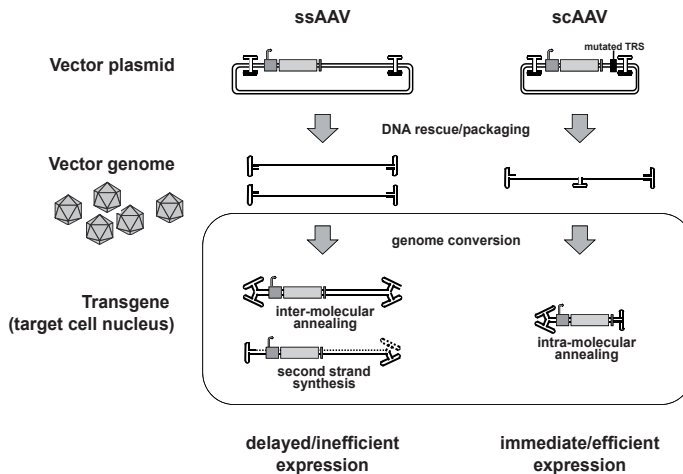


Figure 2. DNA rescue and transduction of a conventional single-stranded AAV (ssAAV) and a self-complementary AAV (scAAV) vector. Full-length ssAAV vector genome of both polarities are rescued from the vector plasmid and individually packaged into the AAV capsids. As a genome conversion in the transduced cell nucleus, the single-to-double stranded conversion of the DNA goes through the inter-molecular annealing or second strand synthesis. In contrast, a scAAV vector with half the size of the ssAAV genome has a mutation in the terminal resolution site (TRS) to form a vector genome with wild-type ITRs at the both ends and mutated ITR at the center of symmetry. After uncoating in the target cell nucleus, this DNA structure can readily fold into transcriptionally active double-stranded form through intra-molecular annealing.

Although various subtypes of the 293 cells harbor the E1 region of the adenovirus type 5 genome, to utilize a 293 cell stably expressing Bcl-xL (293B) has great advantage to support E1B19K function and protect cells from apoptosis.[26] Despite improvements in vector production, including the development of packaging cell lines expressing Rep/Cap or methods to regulate Rep/Cap,[27] maintaining such cell lines remains difficult, as the early expression of Rep proteins is toxic to cells.

We developed a large-scale transfection method of producing AAV vectors with an active gassing system that uses large culture vessels to process labor-effective transfection in a closed system.[28] This vector production system achieved reasonable production efficiency by improving gas exchange to prevent pH drop in the culture medium. Also, vector purification with the dual ion-exchange membrane adsorbers was effective and allowed higher levels of gene transfer *in vivo*. [29] Furthermore, the membrane adsorbers enabled the effective recovery of the AAV vector in the supernatant exosomes of the transduced cells culture.

This rapid and scalable viral purification protocol is particularly promising for considerable *in vivo* experimentation and clinical investigations (Figure 3).

Recent developments also suggest that AAV vector production in insect cells would be compatible with current good manufacturing practice production on an industrial scale.[30]

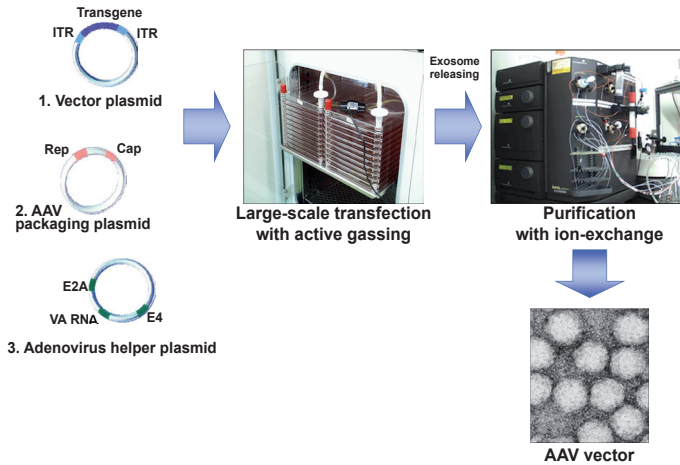


Figure 3. A scalable triple plasmid transfection using active gassing. When (1) a vector plasmid encoding the transgene cassette flanked by ITRs is co-transfected into human embryonic kidney 293 cells with (2) an AAV packaging plasmid harboring *rep-cap* genes and (3) an adenovirus helper plasmid, the AAV vector is produced as efficiently as when using adenovirus infection. A large-scale transduction method to produce AAV vectors with an active gassing system makes use of large culture vessels for labor- and cost-effective vector production in a closed system. Samples containing vector particles are further purified with a quick two-tier CsCl gradient centrifugation and an ion-exchange chromatography to obtain highly purified vector stocks.

2.2. Large-scale production with active gassing

Our protocol utilizes the transfection of 293B cells in one 10-Tray flask (CF10; Nalge Nunc International, Rochester, NY) with a surface area of 6320 cm² by using an active gassing at 500 ml/min. Typical transduction procedure is conducted with one or two CF10 to meet downstream purification protocol. Although previous protocols for recombinant virus production in a large culture vessel had the problem of insufficient transduction efficiency because of inadequate gas exchange, this method to use active gassing significantly improves productivity of the vectors and is linearly scalable from the small 225-cm² flask.[3]

The 293B cells are cultured in Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 (D-MEM/F-12, Invitrogen, Grand Island, NY) with 10% fetal bovine serum (SIGMA-ALDRICH, St Louis, MO), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO₂ incubator. Cells are initially plated at 8×10^7 cells per CF10 to achieve a monolayer of 20 to 40% confluency when cells attached to surface of the flask. The volume of medium

utilized per flask is 1120 ml. Subsequently, cells are grown for 48-72 h until reaching 70-90% confluence and are consequently transfected with appropriate triple plasmids. An aquarium pump (Nisso, Tokyo, Japan) should be used to circulate the gas through the CF10 with 5% CO₂ and humidity in an incubator.

Half of the medium in the CF10 tissue culture flask are exchanged with fresh D-MEM/F-12 containing 10% FBS, 1 h before transfection of the 293 cells. Subsequently, the cells are co-transfected with 650 µg of each plasmid: a proviral vector plasmid, an AAV helper plasmid, as well as an adenoviral helper plasmid, using calcium phosphate co-precipitation. Each plasmid was added to 112 ml of 300 mM CaCl₂. This solution was gently added to the same volume of 2 x HBS (290 mM NaCl, 50 mM HEPES buffer, 1.5 mM Na₂HPO₄, pH 7.0) and gently inverted 3 times to form a uniform solution. This solution was immediately mixed with fresh D-MEM/F-12 containing 10% FBS to produce a homogeneous plasmid solution mixture. Subsequently, the medium in the culture flask was replaced with this plasmid solution mixture. At the end of a 6-12 h incubation, the plasmid solution mixture in the culture flask was replaced with pre-warmed fresh D-MEM/F-12 containing 2% FBS.

2.3. Purification phase

The culture supernatant sample for the ion-exchange procedure is processed by centrifugation and filtration. The culture supernatant fluid 72-96 h after the transduction is sampled and then clarified with an appropriate amount of the activated charcoal (Wako Pure Chemical Industries, Osaka, Japan). Insoluble debris is removed by a centrifugation at 3,000 g for 15 min and filtration. The elucidated culture supernatant is enriched with a hollow fiber cross flow membrane (100,000 NMWC, GE Healthcare, Pittsburgh, PA). For the material obtained from a CF10, 5 mM MgCl₂ (final concentration) with 2,500-5,000 units of Benzonase nuclease is added to incubate for 30 min at 37 °C. Sequentially, 5 mM EDTA (final concentration) is added to terminate the reaction. Place 38 ml of the sample solution in a semi-sterile ultracentrifuge tube (Ultrabottle #3430-3870; Nalge Nunc, Rochester, NY) and remove the cell debris by centrifugation at 10,000g for 15 minutes at 4 °C to achieve cleared lysates. The sample is quickly concentrated by the brief two-tier CsCl (1.25 and 1.60 g/cm³) step gradient centrifugation for 3 h and then the vector fraction is dialyzed in the MHA buffer (3.3 mM MES 3.3 mM HEPES [pH 8.0], 3.3 mM NaOAc).

Chromatography can be performed using an appropriate FPLC system, such as AKTA explorer 10S (Amersham Biosciences, Piscataway, NJ, USA) equipped with a 50 ml Superloop. The sample which passed through the MustangTM S membrane (optional treatment, PALL corporation, NY) is dialyzed against MHA buffer and further loaded onto an anion-exchange membrane (acrodisc unit with MustangTM Q membrane, PALL corporation, equilibrated with MHA buffer) at a rate of 3 ml/min. The membrane is then washed with 10 column volumes of MHA buffer. Bound virus on the MustangTM Q membrane is eluted over a 50 column volume span with a 0-2 M linear NaCl gradient in MHA buffer and 0.5-1 ml fractions are collected. Recombinant rAAV particle number is determined by quantitative PCR of DNase I-treated stocks with plasmid standards. The final titer of the purified vectors

from a CF10 usually ranges around 5×10^{13} genome copies (g.c.), although it depends on the vector constructs and transgene.

3. AAV-mediated therapeutic approach to neuromuscular disease

3.1. DMD gene replacement therapy

Duchenne muscular dystrophy (DMD) is the most common form of childhood muscular dystrophy and is an X-linked recessive disorder with an incidence of one in 3500 live male births.[31] DMD causes progressive degeneration and regeneration of skeletal and cardiac muscles due to mutations in the *dystrophin* gene, which encodes a 427-kDa subsarcolemmal cytoskeletal protein.[32] DMD is associated with severe, progressive muscle weakness and typically leads to death between the ages of 20 and 35 years. Due to recent advances in respiratory care, much attention is now focused on treating the cardiac conditions suffered by DMD patients. The approximately 2.5-megabase *dystrophin* gene is the largest gene identified to date, and because of its size, it is susceptible to a high sporadic mutation rate. Absence of dystrophin and the dystrophin-glycoprotein complex (DGC) from the sarcolemma leads to severe muscle wasting. Whereas DMD is characterized by the absence of functional protein, Becker muscular dystrophy, which is commonly caused by in-frame deletions of the *dystrophin* gene, results in the synthesis of an incompletely functional protein.

Successful therapy for DMD requires the restoration of dystrophin protein in skeletal and cardiac muscles. While various viral vectors have been considered for the delivery of genes to muscle fibers, the AAV-based vector is emerging as an appropriate gene transfer vehicle with the most potential for use in DMD gene therapies. As for another candidate vehicle, the gutted adenovirus vector can package 14-kb of full-length *dystrophin* cDNA due to the large deletion in virus genome. Multiple proximal muscles of seven-day-old utrophin/dystrophin double knockout mice (*dko* mice), which typically show symptoms similar to human DMD, were effectively transduced with the gutted adenovirus bearing full-length murine *dystrophin* cDNA.[33] However, further improvements are needed to regulate the virus-associated host immune response before clinical trials can be performed.

A series of truncated *dystrophin* cDNAs containing rod repeats with hinge 1, 2, and 4 were constructed (Figure 4A).[4] Although AAV vectors are too small to package the full-length *dystrophin* cDNA, AAV vector-mediated gene therapy using a rod-truncated *dystrophin* gene provides a promising approach.[34] The structure and, particularly, the length of the rod are crucial for the function of micro-dystrophin.[35] An AAV type 2 vector expressing micro-dystrophin (DeltaCS1) under the control of a muscle-specific MCK promoter was injected into the tibialis anterior (TA) muscles of dystrophin-deficient *mdx* mice,[36] and resulted in extensive and long-term expression of micro-dystrophin that exhibited improved force generation. Likewise, AAV6 vector-mediated systemic *micro-dystrophin* gene transfer was effective in treating *dko* mice.[37] The potential for ameliorating the pathology of advanced-stage muscular dystrophy by systemic administration of AAV6 vectors encoding a micro-dystrophin expression construct was also demonstrated.[38] Furthermore, AAV9 vector-mediated

micro-dystrophin transduction of *mdx* mice accomplished prevention of cardiac fibrosis as well as heart failure.[23] The transduction efficiency achieved with rAAV9 was nearly complete, with persistent expression for 74 weeks after transduction (Figure 4BC). Both the strong affinity of the rAAV9 for cardiac tissue and the therapeutic effect of the expressed *micro-dystrophin* might be involved in the prevention of the degeneration of the cardiomyocytes and cardiac fibrosis.

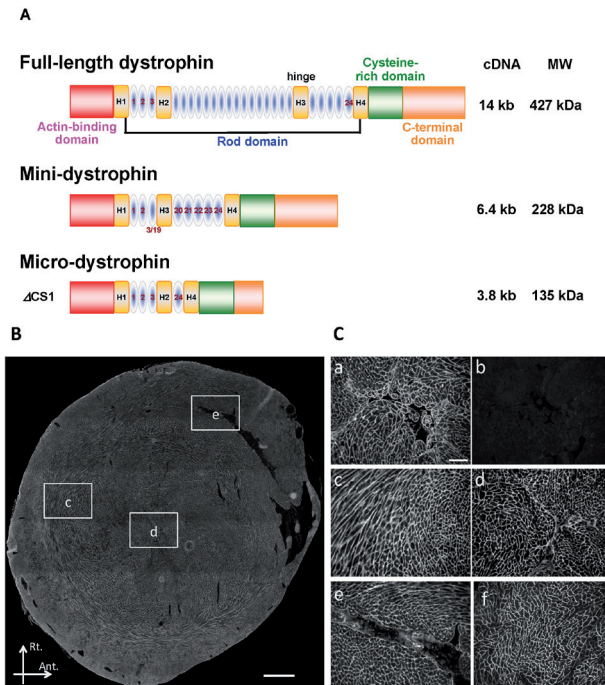


Figure 4. AAV9 vector-mediated cardiac transduction. (A) Structures of full-length and truncated dystrophin. Helper-dependent adenovirus vector can package 14-kb of full-length dystrophin cDNA because of the large-sized deletion in its genome. A mini-dystrophin is cloned from a patient with Becker muscular dystrophy, which is caused by in-frame deletions resulting in the synthesis of partially functional protein. A truncated micro-dystrophin cDNAs harboring only four rod repeats with hinge 1, 2, and 4 and a deleted C-terminal domain (delta C51) is constructed to be packaged in the AAV vector. (B) Transverse section of *mdx* mouse heart at mid-ventricular level 24 weeks after transduction of *micro-dystrophin*, stained with anti-dystrophin antibody NCL-DysB. Scale bar, 500 μm. (C) Expression of dystrophin in C57BL10 hearts at the sarcolemma (a), while it is absent in *mdx* hearts (b). Magnified views of sections from the center of the left ventricle at 28 weeks (c-e) show micro-dystrophin expression in the areas indicated in B (scale bar, 100 μm). At 74 weeks after transduction, *mdx* mice still retain extensive expression of micro-dystrophin (f).

The impact of codon usage optimization on micro-dystrophin expression and function in the *mdx* mouse was demonstrated to compare the function of two different configurations of codon-optimized *micro-dystrophin* genes under the control of a muscle-restrictive promoter

(Spc5-12).[39] Codon optimization of micro-dystrophin significantly increased micro-dystrophin mRNA and protein levels after intramuscular and systemic administration of plasmid DNA or rAAV8. By randomly assembling myogenic regulatory elements into synthetic promoter recombinant libraries, several artificial promoters were isolated whose transcriptional potencies greatly exceed those of natural myogenic and viral gene promoters.[40]

3.2. Intravascular vector administration by limb perfusion

Although recent studies suggest that vectors based on AAV are capable of body-wide transduction in rodents,[21] translating the characteristics into large animals with advanced immune system remains a lot of challenges. Intravascular delivery can be performed as a form of limb perfusion, which might bypass the immune activation of DCs in the injected muscle.[41] We performed limb perfusion-assisted intravenous administration of rAAV8-lacZ into the hind limb of Beagle dogs (Figure 5A).[42] Administration of rAAV8 by limb perfusion demonstrated extensive transgene expression in the distal limb muscles of canine X-linked muscular dystrophy in Japan (CXMD_J) dogs without obvious immune responses for the duration of the experiment over four weeks after injection.

3.3. Systemic transduction and immunological issues

In comparison with fully dystrophin-deficient animals, targeted transgenic repair of skeletal muscle, but not cardiac muscle, paradoxically elicits a five-fold increase in cardiac injury and dilated cardiomyopathy.[43] Because the dystrophin-deficient heart is highly sensitive to increased stress, increased activity by the repaired skeletal muscle provides the stimulus for heightened cardiac injury and heart remodeling. In contrast, a single intravenous injection of AAV9 vector expressing micro-dystrophin efficiently transduces the entire heart in neonatal *mdx* mice, thereby ameliorating cardiomyopathy.[44]

Since a number of muscular dystrophy patients can be identified through newborn screening in future, neonatal transduction may lead to an effective early intervention in DMD patients. After a single intravenous injection, robust skeletal muscle transduction with AAV9 vector throughout the body was observed in neonatal dogs.[45] Systemic transduction was achieved in the absence of pharmacological intervention or immune suppression and lasted for at least six months, whereas rAAV9 was barely transduced into the cardiac muscle of dogs. Likewise, *in utero* gene delivery of full-length murine *dystrophin* to *mdx* mice using a high-capacity adenoviral vector resulted in effective protection from cycles of degeneration and regeneration.[46]

Neo-antigens introduced by AAV vectors evoke significant immune reactions in DMD muscle, since increased permeability of the DMD muscle allows leakage of the transgene products from the dystrophin-deficient sarcolemma of muscle fibers.[47] rAAV2 transfer into skeletal muscles of normal dogs resulted in low levels of transient expression, together with intense cellular infiltration, and the marked activation of cellular and humoral immune responses.[48] Furthermore, an *in vitro* interferon-gamma release assay showed that canine splenocytes respond to immunogens or mitogens more strongly than do murine splenocytes. Therefore, co-administration of immunosuppressants, cyclosporine (CSP) and myco-

phenolate mofetil (MMF) was attempted to improve rAAV2-mediated transduction. The AAV2 capsids can induce a cellular immune response via MHC class I antigen presentation with a cross-presentation pathway,[49] and rAAV2 could also stimulate human dendritic cells (DCs).[50] Whereas the non-immunogenic nature of AAV6 in murine studies, rAAV6 also elicited robust cellular immune responses in dogs.[51] In contrast, other serotypes, such as rAAV8, induce T-cell activation to a lesser degree.[42] The rAAV8-injected muscles showed lowered rates of infiltration of CD4⁺ and CD8⁺ T lymphocytes in the endomysium than the rAAV2-injected muscles.[42]

Resident antigen-presenting cells, such as DCs, myoblasts, myotubes and regenerating immature myofibers, should play a substantial role in the immune response against rAAV. Our study also showed that MyD88 and co-stimulating factors, such as CD80, CD86 and type I interferon, are up-regulated in both rAAV2- and rAAV8-transduced dog DCs (Figure 5B).[42]

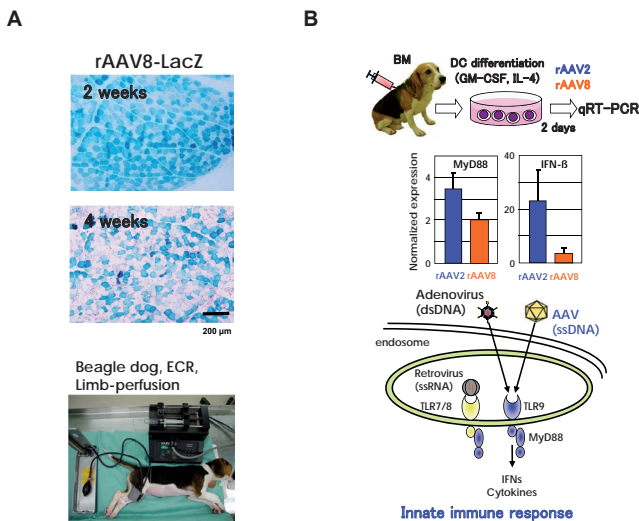


Figure 5. rAAV-mediated transduction of dog. (A) Intravascular vector administration by limb perfusion. A blood pressure cuff is applied just above the knee of an anesthetized CXMD dog. A 24-gauge intravenous catheter is inserted into the lateral saphenous vein, connected to a three-way stopcock, and flushed with saline. With a blood pressure cuff inflated to over 300 mmHg, saline (2.6 ml/kg) containing papaverine (0.44 mg/kg, Sigma-Aldrich, St. Louis, MO) and heparin (16 U/kg) is injected by hand over a 10 second period. The three-way stopcock is connected to a syringe containing rAAV8 (1×10^{14} vg/kg, 3.8 ml/kg). The syringe is placed in a PHD 2000 syringe pump (Harvard Apparatus, Edenbridge, UK). Five minutes after the papaverine/heparin injection, rAAV8-LacZ is injected at a rate of 0.6 ml/sec. Two minutes after the rAAV injection, the blood pressure cuff is released and the catheter is removed. Four weeks after the transduction, the expression slightly fell off. (B) AAV-mediated stimulation of innate immune response via TLR9/MyD88 pathway. Bone marrow (BM)-derived dendritic cells (DCs) were obtained from humerus bones and cultured in RPMI (10% FCS, p/s) for 7 days with canine GM-CSF and IL-4. DCs were transduced with rAAV2- or rAAV8-lacZ (1×10^6 vg/cell for 4 hours, and mRNA levels of MyD88 and IFN- β were analyzed. Untransduced cells were used as a normalization standard to demonstrate relative value of expression. Results are representative of two independent experiments. Error bars represent s.e.m., $n = 3$.

4. Safety and potential impact of clinical trials

4.1. Clinical trials for muscle transduction

While low immunogenicity was considered a major strength supporting the use of rAAV in clinical trials, a number of observations have recently provided a more balanced view of this procedure.[52] An obvious barrier to AAV transduction is the presence of circulating neutralizing antibodies that prevent the virion from binding to its cellular receptor.[53] This potential threat can be reduced by prescreening patients for AAV serotype-specific neutralizing antibodies or by performing therapeutic procedures such as plasmapheresis before gene transfer. Another challenge recently revealed is the development of a cell-mediated cytotoxic T-cell (CTL) response to AAV capsid peptides. In the human factor IX gene therapy trial in which rAAV was delivered to the liver, only short-term transgene expression was achieved and levels of therapeutic protein declined to baseline levels 10 weeks after vector infusion.[52] This was accompanied by elevation of serum transaminase levels and a CTL response toward specific AAV capsid peptides. To overcome this response, transient immunosuppression may be required until AAV capsids are completely cleared. Additional findings suggest that T-cell activation requires AAV2 capsid binding to the heparan sulfate proteoglycan (HSPG) receptor, which would permit virion shuttling into a DC pathway, as cross-presentation.[54] Exposure to vectors from other AAV clades, such as AAV8, did not activate capsid-specific T-cells.

The initial clinical studies lay the foundation for future studies, providing important information about vector dose, viral serotype selection, and immunogenicity in humans. The first virus-mediated gene transfer for muscle disease was carried out for limb-girdle muscular dystrophy type 2D using rAAV1. The study, consisting of intramuscular injection of virus into a single muscle, was limited in scope and the main conclusion was to establish the safety of this procedure in phase I clinical trials. The first clinical gene therapy trial for DMD began in March 2006.[55] This was a Phase I/IIa study in which an AAV vector was used to deliver micro-dystrophin to the biceps of boys with DMD. The study was conducted on six boys with DMD, each of whom received an injection of mini-dystrophin-expressing rAAV2.5 in a muscle of one arm and a placebo in the other arm. Dystrophin-specific T cells were detected after treatment, providing evidence of transgene expression even when the functional protein was not visualized in skeletal muscle.[56] The potential for T-cell immunity to self and non-self dystrophin epitopes should be considered in designing and monitoring experimental therapies for this disease. Basically, this issue is in common with the treatment of genetic diseases. Although concerns regarding risk of an immune response to the transgene product limited the ability to achieve therapeutic efficacy, rAAV2-mediated gene transfer to human skeletal muscle can persist for up to a decade.[57]

4.2. Gene therapy medicine

After more than two decades of expectations, the field of gene therapy appears close to reaching a regulatory approval by proposing rAAV-mediated muscle transduction. European medicine agency eventually recommends first gene therapy medicine for approval.

(<http://www.ema.europa.eu/ema>) The European Medicines Agency's Committee for Medicinal Products for Human Use has recommended the authorization of Glybera (rAAV1-expressing LPL S447X variant) for marketing in the European Union. It is intended to treat lipoprotein lipase deficiency in patients with severe or multiple pancreatitis attacks, despite dietary fat restrictions.

5. Challenges and future perspectives

5.1. Immunomodulation to augment clinical benefits

To regulate host immune response against vectors and transgene products, treatments involving immunosuppressants and other strategies have been attempted in the animal models. A brief course of immunosuppression with a combination of anti-thymocyte globulin (ATG), CSP and MMF was effective in permitting AAV6-mediated, long-term and robust expression of a canine micro-dystrophin in the skeletal muscle of a dog DMD model.[58] To establish the feasibility of multiple AAV1 injections for extending the treatment to whole body muscles, the dystrophic *mdx* mouse was repeatedly transduced with AAV1 vector, and the immune response was characterized.[59] By blocking the T-B crosstalk with anti-CD40 Abs and CTLA4/Fc fusion protein, a five-day-long immunomodulation treatment was found to be sufficient for totally abrogating the formation of anti-AAV1 antibodies.

There have been numerous reports to develop the therapeutic potential of mesenchymal stem cells (or mesenchymal multipotent stromal cells MSCs).[60] Because of their immunomodulatory properties, increasing experimental and early clinical observations indicate that allogeneic, and even xenogeneic, MSCs may be useful for tissue transplantation.[61] In fact, the immune tolerance with MSCs is well investigated in various animal studies. Infusion of syngeneic MSCs into a sensitized mouse model of kidney transplantation resulted in the expansion of donor-specific T-regulatory cells into lymphoid organs, prolonged allograft survival and promoted the development of tolerance.[62]

5.2. Pharmacological intervention

The use of a histone deacetylase (HDAC) inhibitor depsipeptide effectively enhances the utility of rAAV-mediated gene therapy.[63] In contrast to adenovirus-mediated transduction, the improved transduction with rAAV induced by the depsipeptide is due to enhanced transgene expression rather than to increased viral entry. The enhanced transduction is related to the histone-associated chromatin form of the rAAV concatemer in the transduced cells. Since various HDAC inhibitors are approved in clinical usage for many diseases to achieve therapeutic benefits, the application of such inhibitors to the rAAV-mediated gene therapy is theoretically and practically reasonable.

5.3. In situ gene therapy

Transplantation of genetically modified vector-producing cells is a possible future treatment for genetic diseases as an *in situ* gene therapy. MSCs are known to accumulate at the site of inflammation or tumors, and therefore can be utilized as a platform for the targeted delivery of therapeutic agents.[64] The MSCs-based targeted gene therapy should enhance the therapeutic efficacy, since MSCs would deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the reagents act locally without elevating their systemic concentrations. We developed the genetically-modified MSCs that produce viral vectors to augment therapeutic efficacy of systemic gene therapy.[65] MSCs isolated from the SD rats bone marrow were transfected with retroviral vector components by nucleofection. As a result, the injection of luciferase-expressing vector-producing MSCs caused significantly stronger signal of bioluminescence at the site of subcutaneous tumors in mice compared with luciferase-expressing non-vector-producing MSCs. [66] Furthermore, tumor-bearing nude mice were treated with the vector-producing MSCs combined with HSV-*tk*/GCV system to demonstrate improved anti-tumor effects. This study suggests the effectiveness of vector-producing MSCs in systemic gene therapy. The therapeutic benefit of this strategy should be further examined by using rAAV-producing MSCs in the various animal models of inflammatory diseases including neuromuscular disorders.

5.4. Capsid modification

A DNA shuffling-based approach for developing cell type-specific vectors is an intriguing possibility to achieve altered tropism. Capsid genomes of AAV serotypes 1-9 were randomly reassembled using PCR to generate a chimeric capsid library.[67] A single infectious clone (chimeric-1829) containing genome fragments from AAV1, 2, 8, and 9 was isolated from an integrin minus hamster melanoma cell line previously shown to have low permissiveness to AAV. Molecular modeling studies suggest that AAV2 contributes to surface loops at the icosahedral threefold axis of symmetry, while AAV1 and 9 contribute to two-fold and five-fold symmetry interactions, respectively.

A versatile rAAV targeting system to redirect rAAV-mediated transduction to specific cell surface receptors would be useful. Insertion of an IgG binding domain of protein A into the AAV2 capsid at amino acid position 587 could permit antibody-mediated vector retargeting, although producing mosaic particles is required to avoid low particle yields.[68] Alternatively, a targeting system using the genetic fusion of short biotin acceptor peptide along with the metabolic biotinylation via a biotin ligase was developed for the purification and targeting of multiple AAV serotypes.[69]

6. Conclusions and outlook

Although an increasing number of scalable methods for purification of rAAV have been described, in order to generate sufficient clinical-grade vector to support clinical trials we need to further improve a large-scale GMP-compatible system for production and purification. To

translate gene transduction technologies into clinical practice, development of an effective delivery system with improved vector constructs as well as efficient immunological modulation must be established. A novel protocol that considers all of these issues would help improve the therapeutic benefits of clinical gene therapy.

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Applications: Inherited Diseases

Gene Therapy for Primary Immunodeficiencies

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Additional information is available at the end of the chapter

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1. Introduction

Primary immunodeficiencies (PID) are caused by mutations in genes involved in the normal development or activity of the immune system [1, 2]. PIDs include B- and T-cell defects, phagocytic disorders, and complement deficiencies with the common feature of frequent life-threatening infections. The phenotypes vary from asymptomatic (IgA deficiency) to severe PIDs (such as Severe combined immunodeficiencies). Treatment of patients with severe PIDs relies in intravenous injection of immunoglobulins, bone marrow transplantation (BMT) and antibiotics. Identical and haploidentical BMT are the only curative treatment, however, the lack of a HLA-matched donor in over 70% of the patients make necessary the development of new therapeutic strategies [3, 4]. Gene therapy (GT) could be the best alternative for the treatment of patients with severe PID that lack a HLA-matched donor [5]. The aim of GT strategies is the stable correction of the mutated gene on the patient's own haematopoietic stem cells (HSCs).

The first successful gene therapy clinical trial used gamma-retroviral derived vectors expressing common cytokine-receptor gamma chain (γ_c) cDNA in HSCs from X-linked severe combined immunodeficiency (SCID-X1) patients [6]. So far, using a very similar vector platform, over 50 PID patients treated with GT can be considered "cured" from SCID-X1, adenosine deaminase deficiency (ADA) and Wiskott-Aldrich syndrome (WAS) PID [7-13]. However, in six children, GT treatment resulted in clonal T-cell proliferation (leukaemia-like disease) [9].

The results obtained in the SCID-X1, ADA and WAS clinical trials clearly showed the importance to improve vector's safety and efficiency [8,14, 15]. Lentiviral-based vectors have been the vector of choice to enhance efficiency and, at the same time, reduce the side effects of gammaretroviral vectors (see below). Several GT clinical trials for SCID-X1, chronic granu-

lomatous disease (CGD) and WAS PID using lentiviral vectors (LVs) have started in the last few years.

This chapter intend to illustrate the past, present and near future of GT for the treatment of severe PIDs

2. Gamma-retroviral vector based gene therapy clinical trials for primary immunodeficiencies

2.1. Gammaretrovirus-based vectors

Gammaretrovirus, also named oncoretrovirus, are efficient, integrative, easy to manipulate and poorly immunogenic. Vector derived for these retroviruses are often named "retroviral vectors" and "oncoretroviral vectors". All the clinical data that will be presented in this section was obtained using a similar gammaretroviral backbone: **LTR---ψ-----transgene-----LTR**. As consequence the therapeutic gene is expressed through the promoter and enhancer sequences present at the viral LTR. Another common aspect of all the GT strategies presented in this section is the modification of the patient's hematopoietic stem cells (HSCs). However HSCs are quiescent or very slowly dividing cells and gammaretroviral-based vectors require active cell division for transduction [16]. Therefore HSCs transduction protocols require cytokine "pre-stimulation" to induce cell proliferation [17], a process that can modify the characteristics of the haematopoietic precursors [18]. However, since LTR-driven gammaretroviral vectors were the only integrative vectors available at the time, several clinical trials started on SCID-X1, ADA CGD and WAS. An overall conclusion of these clinical trials was that GT is as efficient and safe as haploidentical BMT. However it was also evident the necessity of improving the vector system before GT of PID could be of general use in clinic.

2.2. X-linked Severe Combined Immunodeficiency (SCID-X1)

SCID-X1 is a monogenic disease caused by mutations in the interleukin-2 receptor gamma chain gene (γ c). Patients with SCID-X1 deficiency do not have T nor NK cells, consequently B-lymphocyte function is also intrinsically compromised [19]. SCID-X1 has been an attractive GT target because patient's cells expressing the transgene have a growth advantage over non-expressing cells [20, 21]. Therefore, GT could, in theory, achieved complete immune reconstitution with a relatively low number of gene-corrected cells. The Fischer group at the "Unité d'Immunologie et d'Hématologie Pédiatriques, Hôpital Necker" in France achieved the first unequivocal success of gene therapy in the two patients treated [6]. The authors transduced patients HSCs (CD34⁺) with a Murine Leukaemia Virus (MLV) based vector expressing the γ c cDNA following pre-activation with stem cell factor (SCF), polyethylene glycol-megakaryocyte differentiation factor (PG-MDF), IL-3 and Flt3-L. The continuation of this work and other clinical trials in other countries enrolled a total of 20 SCID-X1 patients [7, 8, 22, 23]. Between 5 and 12 years after GT, 17 of the 20 treated patients are alive and display full or nearly full correction of the T cell deficiency [24, 25]. The GT treatment led to clear benefits since patients

recover from ongoing infections with poor prognosis (disseminated infections) and live in a normal environment without evidence of increased susceptibility to infection.

However, 5 of the 20 patients with SCID-X1 on GT trials developed leukaemia 3-6 years after treatment. Four patients were successfully treated with chemotherapy and they are alive and doing well. However the other patient died from chemotherapy-refractory leukemia [26]. This leukaemia-like disease was a result of vector-mediated up-regulation of host cellular oncogenes (i.e. LMO2) [8, 27]. Several studies have demonstrated that MLV-derived vectors integration favour transcriptionally active genes near transcription start sites (TSSs) [28-30]. Leukemogenesis could also be the result of insertional mutagenesis (activation of the LMO2 oncogene) combined with the acquisition of genetic abnormalities unrelated to vector insertion, such as the increase activity of NOTCH1 or the deletion of CDKN2A gene [8].

However, in spite of the secondary effects observed, the results obtained with GT using first generation MLV-based vectors are comparable to those obtained with HLA-identical HSC transplant (HSCT). It is expected that next generation vectors will certainly improve these results as it will discussed later.

2.3. Adenosine Deaminase (ADA) Severe Combined Immunodeficiency (ADA-SCID)

ADA-deficiency has been also considered an important target for GT. The *ADA* gene codify for an enzyme that is expressed in all tissues and catalyses the deamination of 2'-deoxyadenosine and adenosine to 2'-deoxyinosine and inosine. Its absence or malfunction cause the accumulation of purine metabolites that are toxic to the cells. Although the *ADA* gene is expressed in all tissues, the accumulation of purine metabolites in the immune cells is the main problem. As consequence, ADA patients suffer from lymphopenia, reduced (or absent) cellular and humoral immunity, failure to thrive and recurrent infections. Additionally, the accumulation of purine metabolites in other tissues also produces skeletal, hepatic, renal, lung, and neurologic abnormalities [31, 32]. Like for SCID-X1, bone marrow transplantation (BMT) is the best therapeutic alternative. However, contrary to SCID-X1, there are other treatment options that allow ADA patients to have near-normal lives: Enzyme replacement therapy (ERT) with polyethylene-glycol-conjugated bovine ADA (PEG-ADA). However, although ERT treatment is well tolerated and can partially restore immune function, its effect decline over time and, in addition, lifelong treatment is very expensive[33].

ADA deficiency has been successfully treated by GT using a similar approach to that for SCID-X1, but requiring mild bone-marrow chemoablation [34]. The authors showed immunological and metabolic reconstitution after transplantation of gene-modified CD34⁺ using ADA-expressing-MLV based vectors. The selective growth advantage of ADA-expressing lymphocytes played an important role in the success of this trial. Similar findings have been reported by Gaspar *et al.* [23] and again by Aiuti *et al*[10]. In total, over 40 patients with ADA have been treated in Italy, UK and USA. At present all patients are alive and 29 of them do not require ERT [9, 10, 23, 25, 34-36].

It is important to remark that no leukaemia-like disease have been observed in the ADA-SCID GT trial. The author propose that the differences between SCID-X1 and ADA might be related

with SCID-X1 genetic background or the role of the therapeutic transgene (*ADA* is a house-keeping enzyme whereas γ_c is a potential oncogene growth factor receptor). However, in the last clinical trial some non-life threatening adverse effects have been reported such as neutropenia (2 patients), treatment-related infections (2 patients), Epstein-Barr virus reactivations (1 patient) and autoimmune hepatitis (1 patient).

2.4. X-linked Chronic Granulomatous Disease (X-CGD)

Chronic granulomatous disease (CGD) is a rare PID characterized by severe, life threatening bacterial and fungal infections. Patients with CGD have also defective degradation of inflammatory mediators leading to granuloma formation. All of these defects are caused by mutations in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits in phagocytic cells [37]. *gp91^{phox}* mutations occur in up to 70% of the CGD cases and represent the X-linked form of this disorder (X-CGD). Neutrophils, monocytes, macrophages, and eosinophils from CGD patients cannot generate superoxide and other reactive oxygen intermediates to destroy invading bacteria and fungi.

Contrary to SCID-X1 and *ADA*, CGD is a difficult target for GT, since the expression of the correct form of the gene does not provide selective advantage to hematopoietic progenitors. In addition, myeloid cells have a short life span and therefore a large amount of HSC must be corrected to achieve clinical benefits. Myeloablative conditioning is therefore required to increase the amount of gene-modified cells that engraft into the patients. Several GT clinical trials for CGD have been conducted since 1997. Initial studies using retroviral vector to express *p47-phox* into CD34+ cells, resulted in low and short-term engraftment of CGD-corrected cells [38]. More recent GT clinical trials on X-CGD conducted in Franckfurt, Zurich, London, USA and Seoul resulted in higher correction and clinical benefit in several patients. Dr Grez's group showed the most dramatic effects in two children (5 and 8 years old) showing recovery from severe pulmonary and spinal aspergillosis. GT treatment also achieved recovery from paraparesis of both legs in one of the children [39]. However, the efficacy was only partial due to a progressive loss of gene-corrected cells over time [39-41]. The loss of transgene expression was, at least in part, due to inactivation of the vector promoter. However, there are other hypothesis that point to the potential toxicity of ectopic expression of *gp91* gene on HSCs as a potential cause of the loss of gene-corrected cells [42]. In addition, three patients developed a myelodysplastic syndrome (MDS) due to transactivation of the MDS/EVI oncogene by the retroviral enhancer [40]. The MDS was fatal for two of the patients while the third was treated with HSCTs. These results revealed the importance of developing new, safer and more efficient vectors for GT in CGD.

2.5. Wiskott-Aldrich Syndrome (WAS)

Wiskott-Aldrich syndrome (WAS) is a X-linked PID caused by mutation in the *WAS* gene coding for the Wiskott-Aldrich syndrome protein (WASP), a hematopoietic-specific member of regulators of the actin cytoskeleton [43, 44]. The most severe form of WAS (where the mutation cause total absence of protein or function) is characterized by recurrent infections,

microtrombocytopenia, eczema and higher susceptibility to autoimmune diseases and lymphoid malignancies [45].

As for other PID, HLA-identical sibling HSC donor transplantation is considered the treatment of choice (over 80% survival rate). Allogeneic HSCTs is offering nowadays good outcomes due to improvements in HLA-typing and new alternative donor sources and myeloablative conditioning regimens [46]. However, patients lacking a HLA-matched donor still require alternative therapeutic approaches. In this direction GT could be an alternative in the near future for these patients. In fact WAS is an attractive target for GT since expression of WASP confer selective growth advantage [47-52].

Dr Klein group (Hannover Medical School, Hannover, Germany) performed the first clinical trial for WAS GT [53]. 10 patients were enrolled in this trial and they received autologous CD34⁺ cells transduced with LTR-driven gammaretroviral vectors expressing WASP. All patients received reduced intensity conditioning with Busulfan. Most of the patients treated gain WASP expression in multiple lineages. Platelet counts increased and clinical condition improved with resolution of eczema and bleeding disorder [54, 55]. However, as occurred in the SCID-X1 clinical trials, four out of 10 of the treated patients developed leukaemia [55, 56]. The presence of the strong LTR enhancer and the patient's predisposition to develop lymphomas could favour the high frequency of leukaemia in this trial.

3. Lentiviral-vector based gene therapy clinical trials for primary immunodeficiencies

As soon as the first cases of leukaemia appeared in the SCID-X1 GT trial, it was clear that LTR-driven gammaretroviral vectors were not the vector of choice to go further into clinic. Improvements in the gammaretroviral vectors and the design of new integrative vectors became the main goal in the GT field. Several groups have dedicated considerable effort to understand the mechanism of leukomogenesis upon gammaretroviral transduction. The LMO2 oncogene was found in 4/5 cases in the SCID-X1 trial and it is now clear that retrovirus-mediated gene transfer can deregulate proto-oncogene expression through the LTR enhancer activity. With this in mind, Dr. Naldini's group have developed self-inactivated (LTR mutated) lentiviral vectors (based in HIV-1) which have one of the best efficiency/safety ratio [57-59]. LVs, contrary to gammaretroviral vectors are able to achieve efficient transduction of HSCs with minimal activation [60]. They are also safer than gammaretroviral vectors due to their less genotoxic integration site [61-63]. Several clinical trials for PID have started using HIV-1-based vectors and some promising results have already been shown on international meetings. In most cases, the general structure of the vectors is as follow: **LTRAU3-- ψ ----human promoter -----transgene----- LTRAU3**

There are at the moment two GT clinical trials on going for SCID-X1 using lentiviral vectors (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>). One is designed for newly diagnose children (St Jude Children's Research Hospital) and other is a Phase I/II non-randomized clinical trial designed to treat 13 patients with SCID-X1 who are between 2 and 30 years of age

and who have clinically significant impairment of immunity. Both cases are based on mice experiments showing a better profile of lentiviral vectors both in term of reconstitution and safety [64].

Dr Gaspar and Dr Kohn have launched two other clinical trials using lentiviral vectors to treat ADA patients in UK and USA respectively. Both groups use EF1 promoter driven lentiviral vectors produced at the same site (Indiana University Vector Production Facility) through a Transatlantic Gene Therapy Consortium. The primary objective of the trial is to examine the safety of the protocol in 10 patients transplanted with LV gene-modified CD34⁺ cells. The protocol will involve non-myeloablative conditioning with busulfan and withholding of PEG-ADA ERT. As secondary objectives the trial will aim for the expression of ADA in peripheral blood leucocytes and immune reconstitution.

CGD is probably the PID where the necessity to improve vector efficiency and safety has been more obvious. The absence of the selective advantage of the gene-modified cells and the short life span of myeloid cells reduce the clinical benefits of gammaretroviral vectors but kept all the secondary effects. In addition, the potential toxicity of ectopic expression of gp91^{phox} on HSCs required the use of physiologically regulated vectors [65] expressing the transgene specifically in granulocytes. Very encouraging results have been obtained in animal models using transcriptionally regulated LV [66, 67]. The first clinical trial for CGD using LV started on November 2011 directed by Adrian Thrasher at Great Ormond Street Hospital for Children (UK). The primary outcome measures will be overall survival but the trial will also study reduction in frequency of infections and long-term immune reconstitution (<http://clinicaltrials.gov/ct2/show/NCT01381003>).

As SCID-X1 and CGD, GT for WAS has also good reasons to change the therapeutic vectors (see above). There are four clinical trials on going for WAS using LV (FR-0047, UK-0168 and US-1052: journal of gene medicine GT clinical trials data base; NCT01515462: Clinicaltrial.gov). All trials will use a similar construct which drive the expression of the WASP cDNA through its own promoter. The WASP-promoter-driven LVs are haematopoietic-specific [47, 49, 68], physiological [49, 69] and avoid deleterious effects of over-expression in non-target cells[70]. Preliminary data presented at the 20th European Society of Gene and Cell Therapy by the Italian and French groups showed impressive results both, in terms of immune reconstitution and safety profile. It is important to note that integration site analysis in these patients did not show any preference for the proto-oncogens LMO2 or EVI1. In addition they didn't observe, at the time of analysis, any evidence of clonal dominance (usually indicative of proto-oncogenes activation).

4. Future directions

Based on the data shown, it does appear that new generation LVs driving the expression of the transgene through physiological promoters could be a big step toward GT clinical translation. Exciting results are expected on the clinical trials undergoing at the moment. Still, LV integrates randomly at active sites in the cell genome and can therefore alter its normal

expression pattern. New, undesired side effects could appear in the future. New vectors must still consider improving two safety aspects: 1- genotoxicity (genomic alteration due to vector integrations) and 2- ectopic/unregulated expression of the transgene. Strategies to minimize or eliminate genotoxicity problems can be grouped in those based in improving retroviral vectors and those based in the development of non-viral technologies such as gene editing (revised in [14, 65]).

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Gene Therapy for Diabetic Retinopathy – Targeting the Renin-Angiotensin System

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Diabetic retinopathy clinical features and current treatment options

The prevalence of diabetes has been continuously increasing for the last few decades and it is being recognized as a worldwide epidemic [1]. Diabetic retinopathy (DR) is the most common diabetic microvascular complication, and despite recent advances in therapeutics and management, DR remains the leading cause of severe vision loss in people under age of sixty [2-4]. The prevalence of DR increases with duration of diabetes, and nearly all individuals with type 1 diabetes and more than 60% of those with type 2 have some form of retinopathy after 20 years [5-7].

Diabetic retinopathy (DR) is characterized by the development of progressive pathological changes in the retinal neuro-glial cells and microvasculature. The earlier hallmarks of diabetic retinopathy include breakdown of the blood-retinal barrier (BRB), loss of pericytes, thickening of basement membrane, and the formation of microaneurysms, which are outpouchings of capillaries [8]. BRB breakdown results in increased vascular permeability and leakage of fluid into the macula causing macular edema, another significant cause of vision loss in those with diabetes. With the progression of diabetic retinopathy, hemorrhage, macular edema, cotton wool spots, all signs of retinal ischemia, and hard exudates, the result of precipitation of lipoproteins and other circulating proteins through abnormally leaky retinal vessels become increasingly apparent. More severe and later stages of diabetic retinopathy, known as proliferative diabetic retinopathy (PDR), is char-

acterized by pathological neovascularization. Vision loss can occur from vitreous hemorrhage or from tractional retinal detachment [8, 9].

Despite recent developments in the pharmacotherapy of DR, treatment options for patients with DR are still limited. Laser photocoagulation, the primary treatment option for patients with PDR, is still considered gold standard therapy for the treatment of PDR. Although this treatment slows the loss of vision in those with PDR, it does not represent a cure, and is in itself a cell destructive therapy. Corticosteroids and anti-VEGF agents have shown promising results with regard to prevention of neovascularization, but remain limited in use due to their short-duration effects. More importantly, none of these agents have been able to substitute for the durability and effectiveness of laser mediated panretinal photocoagulation in preventing vision loss in the late stages of DR.

1.2. RAS and diabetic complications

The renin-angiotensin system (RAS) plays a vital role in the cardiovascular homeostasis by regulating vascular tone, fluid and electrolyte balance, and in the sympathetic nerve system. Angiotensin II (Ang II), a peptide hormone of RAS, has been known to regulate a variety of hemodynamic physiological responses, including fluid homeostasis, renal function, and contraction of vascular smooth muscle [10]. In addition, Ang II is capable of inducing a multitude of non-hemodynamic effects, such as the induction of reactive oxygen species (ROS), cytokines, and the stimulation of collagen synthesis [11-14]. Most of the pathophysiological actions of Ang II are mediated via activation of Ang II type 1 receptors (AT1R), G protein-coupled receptors (GPCRs) that couple to many signaling molecules, including small G proteins, phospholipases, mitogen-activated protein (MAP) kinases, phosphatases, tyrosine kinases, NADPH oxidase, and transcription factors to stimulate vascular smooth muscle cell growth, inflammation, and fibrosis [11, 15, 16]. Dysregulation of RAS has been implicated in a number of major cardiovascular and metabolic diseases, including endothelial dysfunction, atherosclerosis, hypertension, renal disease, diabetic complications, stroke, myocardial infarction and congestive heart failure [17, 18]. RAS blockade produces beneficial cardiovascular and renal effects in numerous clinical trials [19-21].

1.3. Recent advances in RAS research

Recent discoveries have revealed that the RAS hormonal signaling cascade is more complex than initially conceived with multiple enzymes, effector molecules, and receptors that coordinately regulate the effects of the RAS. Recent studies have identified additional peptides with important physiological and pathological roles, new enzymatic cascades that generate these peptides and more receptors and signaling pathways that mediate their function [22, 23].

Discovery of angiotensin-converting enzyme 2 (ACE2) has resulted in the establishment of a novel axis of the RAS involving ACE2/Ang-(1-7)/Mas [24-27]. ACE2, like ACE, is a zinc-metallopeptidase, exhibiting approximately 42% amino acid identity with ACE in its catalytic domain. However, unlike somatic ACE, ACE2 only contains a single catalytic site and func-

tions as a carboxymonopeptidase, cleaving a single C-terminal residue from peptide substrates, thus ACE2 is able to cleave Ang II to form Ang (1-7). Ang (1-7), a biologically active component of the RAS [28-30] binds to a G-protein coupled receptor, Mas receptor [31], and plays a counter-regulatory role in the RAS by opposing the vascular and proliferative effects of Ang II [32]. A current view of RAS consists of at least two axis with counteracting biologic effects (Figure 1).

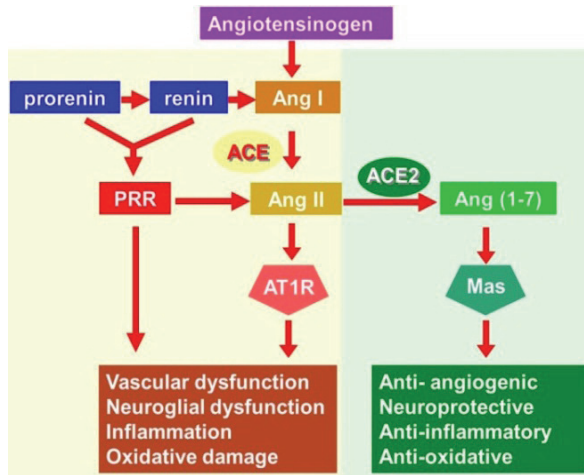


Figure 1. Schematic diagram depicting the key components of the Renin Angiotensin System. Angiotensinogen is cleaved by renin to form angiotensin I (Ang I). Angiotensin converting enzyme (ACE) converts Ang I into Angiotensin II (Ang II) the main effector peptide of the RAS. Ang II elicits cellular effects by activating the main receptor, Angiotensin II receptor 1 (AT1R), as well as other receptors (not shown). Angiotensin II-converting enzyme 2 (ACE2), a recently discovered component of RAS, cleaves Ang II to form Angiotensin (1-7) (Ang 1-7), which activate Mas receptor to produce counteracting effects mediated by Ang II. All these components are expressed locally in various cell types in the eye, regulating metabolism, cell survival, and other local neuronal-vascular and immune-modulating functions in the retina.

This vasoprotective axis of RAS counteracts the traditional proliferative, fibrotic, proinflammatory and hypertrophic effects of the ACE/Ang II/AT1R axis of the RAS [24]. The importance of the vasodeleterious axis of the RAS [ACE/angiotensin II (Ang II)/ AT1R] in cardiovascular disease, as well as in diabetes and diabetic complications, is well established since ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs) are leading therapeutic strategies [20, 33-35]. However, the impact of the vasoprotective axis of the RAS remains poorly understood [24, 36-38]. The concept that shifting the balance of the RAS towards the vasodilatory axis by activation of ACE2 or its product, Ang-(1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis [24, 39-43]. Indeed, ACE2/Ang-(1-7) activation is now considered to be a critical part of the beneficial actions of ACEi and ARB drugs [24, 36].

1.4. Tissue RAS in end-organ damage

The classical (endocrine) RAS has been traditionally regarded as systemic hormonal system. Ang II is formed from liver-synthesized angiotensinogen via a series of proteolytic cleavage events. Circulating Ang II activates AT1 and AT2 receptors in various tissues, such as the brain, adrenal and vascular tissues to modulate cardiovascular and hydro-mineral homeostasis.

However, most components of RAS have also been identified in essentially every organ including kidney, heart, liver, brain, adipose tissue, reproductive tissue, hematopoietic tissue, immune cells and eye, and increasing evidence supports the existence of tissue- specific RAS that exerts diverse physiological effects locally and independently of circulating Ang II [44-46]. These tissue- specific paracrine, intracrine and autocrine actions of RAS may contribute to end-organ damage in many pathological conditions including diabetic complications and maybe the basis for the reported limited beneficial effects of RAS blockade.

2. Ocular RAS in pathogenesis of diabetic retinopathy

Increasing evidence continues to implicate the involvement of the local renin-angiotensin-system (RAS) in retinal vascular dysfunctions. Various components of RAS have been detected in the different cell types of the eye (Table 1).

RAS components	Retinal Localization	Reference
Angiotensinogen	Retinal microvasculature, RGCs, RPE	[47, 48]
Angiotensin I	Aqueous, vitreous, and subretinal fluid	[49]
Angiotensin II	Aqueous, vitreous, and subretinal fluid, RGCs, retinal endothelial cells and photoreceptors	[49-51]
Angiotensin 1-7	Muller cells	[50]
Renin	Muller cells and vitreous fluid	[52, 53]
Renin receptor	Retinal microvasculature, microglia, astrocytes, RGCs, RPE	[54-58]
ACE	Muller cells, RGCs, retinal endothelial cells, photoreceptors, and vitreous	[51, 59-61]
ACE2	Retina	[50]
AT1R	Muller cells, retinal blood vessels, photoreceptors and RGCs	[50, 51]
AT2R	Muller cells, nuclei of some inner, nuclear layer neurons, and ganglion cells	[50]
Mas receptor	RGCs, retinal microvasculature, microglia, subset of astrocytes	unpublished results

GC: retinal ganglion cells; RPE: retinal pigment epithelium.

Table 1. All components of RAS are expressed locally in the eye.

Hyperglycemia has been shown to directly stimulate angiotensin gene expression via the hexomerase pathway, thus contributing to increased Ang II synthesis [62]. Elevated levels of renin, prorenin, and Ang II have been found in patients with DR. In fact, ACE inhibitors and angiotensin receptor blockers (ARBs) have been shown to improve diabetes-induced vascular, neuronal, and glial dysfunction [61, 63–66]. Recent clinical studies have also clearly demonstrated the beneficial effects of RAS inhibition in both type 1 and type 2 diabetic patients with retinopathy [67–71]. Despite these positive outcomes, RAS blockers are not completely retinoprotective and retinopathy still progresses to more advanced stages. This could be attributed to the existence of local Ang II formation and that current therapeutic agents are unable to cross the blood-retina barrier (BRB) in a concentration sufficient to influence the local RAS in the eye. In addition, increasing evidence suggests that Ang II can be generated via multiple pathways, many of which may not be blocked by classic inhibitors of ACE [72–75]. Furthermore, additional components of RAS that contribute to end-organ damage, such as receptors for renin and prorenin (PRR), have been recently identified [76]. Activation of prorenin/PRR signaling pathway can initiate the RAS cascade independent of Ang II [76].

Ang II may contribute to development and progression of DR by several mechanisms. First, Ang II has been shown to increase VEGF expression directly via activation of AT1R signaling and indirectly by PCK activation [77] to enhance the role of VEGF induced vascular permeability and angiogenesis. Treatment with ACE inhibitors reduces vitreous levels of VEGF and attenuates VEGF-mediated BRB breakdown [78, 79]. Second, Ang II, mediated via AT1R, also contributes to diabetes-induced retinal inflammation by activation of nuclear factor- κ B signaling pathway within retinal endothelial cells [80, 81] leading to the release of inflammatory cytokines which perpetuates the inflammatory cycle. Pro-inflammatory cytokines, chemokines and other inflammatory mediators play an important role in the pathogenesis of DR [82, 83]. These lead to persistent low-grade inflammation, the adhesion of leukocytes to the retinal vasculature (leukostasis), breakdown of BRB and neovascularization with subsequent sub-retinal fibrosis or disciform scarring [84–88]. Third, Ang II may contribute to increased oxidative stress in diabetic retina. Ang II induces reactive oxygen species (ROS) production by activation of NADPH oxidases [89], which has been implicated in diabetic complications [90, 91]. Ang II also induces mitochondrial ROS production, which further stimulate of NADPH oxidases leading to vicious cycle and contributing tissue damage [92, 93].

Fourth, Ang II may also contribute to neuronal dysfunction induced by diabetes [94]. Receptors for Ang II are also expressed in the inner retinal neurons (Table 1). Ang II induced AT1R signaling may cause neuronal dysfunction by reducing the synaptophysin protein in the synaptic vesicles [94].

3. Protective role of the ACE2/Ang1-7-Mas axis of RAS in diabetic complications

The discovery of ACE2-mediated degradation of Ang II into the protective peptide Ang 1-7 thereby negatively regulating the classic RAS, has instigated stimulated interest regarding the potential of ACE2 as a therapeutic target [88, 89], and strategies aimed at enhancing

ACE2 action may have important therapeutic potential for cardiovascular disorders as well as for diabetic complications [40, 95-99]. Ang (1-7) has been shown to prevent diabetes-induced cardiovascular dysfunction [100] and nephropathy [101]. The protective effect of Ang 1-7 signaling is at least in part mediated by direct inhibition of diabetes-induced ROS production due to elevated NADPH oxidase activity [101, 102] and reduction in PPAR-gamma and catalase activities [102]. Adenovirus mediated gene delivery of human ACE2 in pancreas improved fasting blood glucose, beta-cell dysfunction and apoptosis occurring in type 2 diabetes mouse model [103]. The importance of ACE2 as a negative regulator of RAS in diabetic complications is supported by the facts that ACE2 deficiency exacerbates diabetic complications [104, 105] and enhancing ACE2 action counteracts the deleterious effects of Ang II and produces protective effects [96-99, 106].

3.1. Diabetes induced changes in the expression of the retinal RAS genes in the mouse retina during the progression of diabetes

We have previously shown that diabetes induced by STZ treatment in eNOS^{-/-} mice results in more severe, accelerated retinopathy than diabetes in untreated eNOS^{+/+} animals [107]. Thus it became critical to compare retinal mRNA levels of the RAS genes in control and diabetic animals during the progression of diabetes. We observed significant (3-10 fold) increases in the mRNA levels of the vasodeleterious axis of the RAS (angiotensinogen, renin, pro/renin receptor, ACE and AT1 receptor subtypes) following STZ treatment (Figure 2) [108]. In contrast, there was ~ 30% reduction in ACE2 mRNA following an initial stimulatory response. As a result the ACE/ACE2 mRNA ratio was increased by 10-fold, while AT1R/Mas ratio was increased by 3-fold following one month of diabetes (Figure 2). These observations were our initial indication that DR is associated with a shifting balance of the retinal RAS towards vasodeleterious axis.

3.2. Enhancing ACE2/Ang1-7-Mas axis by AAV-mediated gene delivery

3.2.1. Characterization of AAV vectors expressing ACE2 and Ang-(1-7)

AAV vector expressing the secreted form of human ACE2 was constructed under the control of the chicken-beta-actin (CBA) promoter (Figure 3A). This secreted form of ACE2 has been previously characterized and shown to be active enzymatically [109]. Since Ang-(1-7) peptide contains only 7 amino acids and small peptides are usually difficult to express in mammalian cells, we designed an expression construct in which the Ang-(1-7) peptide is expressed as part of the secreted fusion GFP protein, and is subsequently cleaved upon secretion into the active peptide. Expression of the fusion sGFP-FC-Ang-(1-7) is under the control of the CBA promoter in the AAV vector (Figure 3A) and was confirmed by transfecting HEK293 cells using this plasmid DNA (Figure 3B). To ensure that the fusion protein was indeed secreted, proteins isolated from the culture supernatants as well as cell lysates from transfected, sham-transfected or untransfected cells were analyzed by western blotting (Figure 3B). Mass spectrometry analysis of Ang (1-7) peptide in supernatant samples of HEK293 cells transfected with the sGFP-FC-Ang-(1-7) plasmid DNA was also performed. The Ang-(1-7) peptide is detectable in supernatant isolated from cells transfected with sGFP-FC-Ang-(1-7) plasmid DNA, but not detectable

in samples isolated from un-transfected cells, or cells transfected with the control plasmid expressing only the cytoplasmic GFP protein (data not shown). Intravitreal administration of AAV-Ang-(1-7) resulted in a robust transduction of retinal cells primarily within the inner retinal layer (Figure 3C-F). This was associated with an increase in both cellular and secreted Ang-(1-7) (Figure 3G-H). Similarly, ACE2 protein level was increased in the retina following transduction with AAV-ACE2 (Figure 3G).

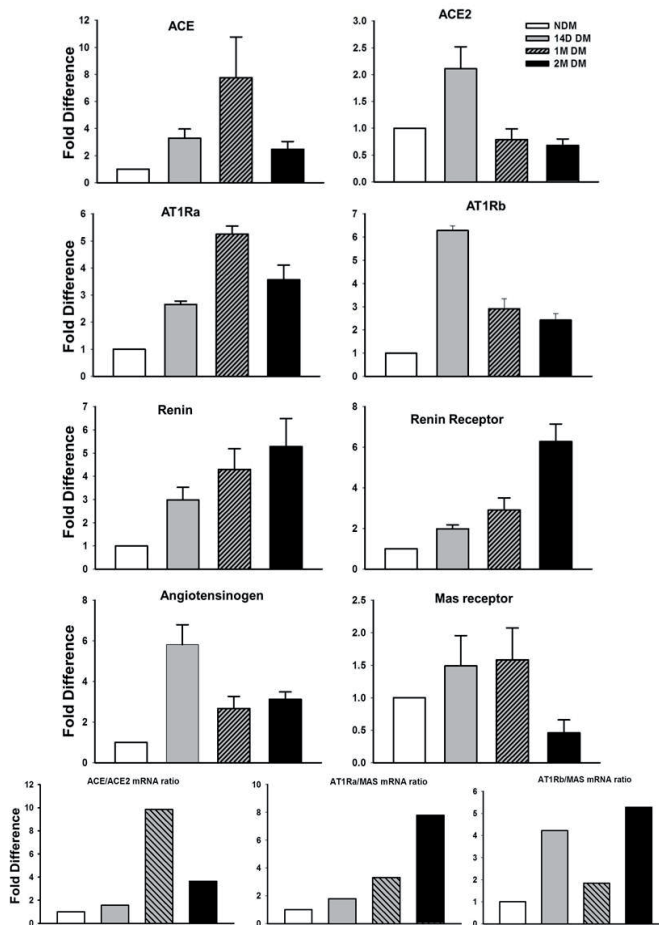


Figure 2. Real-time RT-PCR analysis of retinal mRNA for renin-angiotensin system genes. Values represent fold difference compared to age matched non-diabetic retinal samples for each gene at each time point (14 day and 1 month after induced diabetes). DM: diabetic. NDM: non-diabetic. At least 4 eyes were analyzed at each time point. * $p < 0.01$ (versus NDM group). (From [108] with permission of Mol. Therapy).

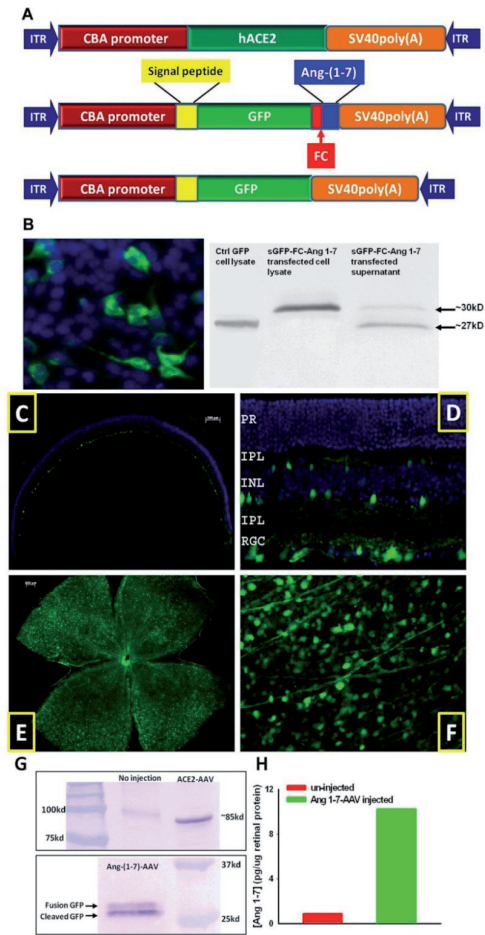


Figure 3. Construction and characterization of AAV vectors expressing ACE2 and Ang-(1-7).A: Maps of the AAV vector expressing the human ACE2 gene (hACE2) and the AAV vector expressing Ang-(1-7) gene. The Ang-(1-7) peptide is expressed as part of fusion protein, and cleaved in vivo upon secretion at the furin cleavage (FC) site. ITR: inverted terminal repeat; CBA: CMV- chicken-β-actin promoter. A control vector contains the coding region for the secreted GFP without the Ang-(1-7) peptide coding sequence. B: Expression and cleavage of the fusion protein. In cultured HEK293 cells transfected with the plasmid sGFP-FC-Ang-(1-7), or infected with AAV-sGFP-FC-Ang-(1-7), there was robust expression of GFP as expected. Proteins isolated from cell lysates contained a single protein band with molecular weight ~30 kd, as predicted for the precursor (fusion protein), but culture supernatants contained two protein bands (30kd and a 27kd), indicating that the secreted protein is cleaved at the furin cleavage site as predicted. C-F: Transduction of mouse retina with AAV vector expressing sGFP-FC-Ang-(1-7) and hACE2. A single intravitreal injection of 1μl AAV vector (10⁹ vg/eye) resulted in efficient transduction of inner retinal cells, primarily retinal ganglion cells. C. Low magnification of cross section of a mouse eye that received AAV2-sGFP-FC-Ang-(1-7) injection. D. Higher magnification of mouse retina showing GFP expression. E. A retinal whole mount showing GFP expression. F. Higher magnification of the same retinal whole mount. G: Western blot of proteins isolated from an uninjected eye and an eye injected with AAV2-ACE2 (top)

and AAV2-sGFP-FC-Ang-(1-7) (bottom) compared to a molecular weight standard (right lane). H: Ang-(1-7) peptide levels in the retina with and without AAV-sGFP-FC-Ang-(1-7) injection. There was more than a 10-fold increase in Ang-(1-7) peptide level detected by using an Ang-(1-7) specific ELISA kit (Bachem, San Carlos, CA) in retinas receiving injection of AAV-sGFP-FC Ang-(1-7). PR: photoreceptor; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC: retinal ganglion cells. (From [108] with permission of Mol. Therapy).

3.2.2. Ocular gene delivery of ACE2/Ang-(1-7) via the AAV vector in the retina results increased ACE2 activities and Ang-(1-7) peptide levels

Diabetes induced more than a 5-fold increase in ACE activity in the retinas of eNOS^{-/-} mice, whereas ACE2 activity was relatively unchanged (Figure 4A). AAV2-ACE2 injected retinas show more than a two-fold increase in ACE2 enzymatic activity (Figure 4A) and this is associated with a reduced level of Ang II and increased Ang-(1-7) peptide level (Figure 4B), but has only a marginal effect on ACE activity (Figure 4A). Injection of AAV2-Ang-(1-7) has no effect on ACE2 activity, but significantly decreased ACE activity (Figure 4A).

We also determined Ang II and Ang-(1-7) peptide levels using a commercial EIA kit (Bachem, San Carlos, CA). STZ induced diabetes resulted in more than a 2-fold increase in Ang II levels whereas the Ang-(1-7) level was unchanged in the retinas of eNOS^{-/-} mice (Figure 4B). This increase of Ang II was completely normalized in retinas injected with AAV-ACE2 but was unchanged in retinas injected with AAV-Ang-(1-7) vector (Figure 4B).

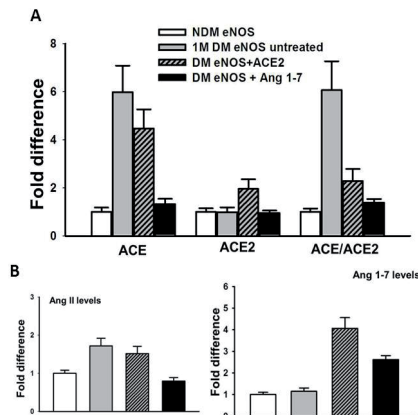


Figure 4. ACE, ACE2 activities and angiotensin peptide levels in the mouse retina. A: ACE and ACE2 enzymatic activities and ACE/ACE2 ratios in non-diabetic (NDM), 1 month diabetic (1M DM), and 1 month diabetic eNOS^{-/-} mouse retinas treated with AAV-ACE2/Ang-(1-7). Values are expressed as fold differences compared with age-matched non-diabetic group. *p<0.01 (versus untreated DM group, N=6/group). B: Ang II and Ang-(1-7) peptide levels in non-diabetic (NDM), 1 month diabetic (1M DM), and 1 month diabetic eNOS^{-/-} retinas treated with AAV-ACE2/Ang-(1-7), measured by ELISA using a commercial kit. *p<0.01 (versus untreated DM group). Values represent fold difference compared with age-matched non-diabetic group. Three retinas were pooled for each measurement, each measurement was done in duplicates, and three separate pools were averaged for each group. (From [108] with permission of Mol. Therapy).

3.3. Protective role of ACE2/Ang (1-7) AAV gene delivery in mouse model of DR

3.3.1. Enhanced ACE2/Ang1-7 expression in the retina reduced diabetes-induced retinal vascular leakage

We investigated if elevated expression of retinal ACE2 or Ang-(1-7) would overcome the vasodeleterious effect of the ACE/AT1R axis and prevent the development of diabetes-induced retinopathy. Effects of increased ACE2 and Ang-(1-7) expression on retinal vascular permeability were evaluated by FITC-labeled albumin extravasations and quantified by measuring its fluorescence intensity in serial sections from non-diabetic, untreated, ACE2 treated diabetic eNOS^{-/-} mice and Ang 1-7 treated diabetic eNOS^{-/-} mice. Induction of diabetes for 2 month in eNOS^{-/-} mice resulted in a 2-fold increase in vascular permeability. This pathophysiology was significantly reduced in diabetic retinas which received ACE2/Ang-(1-7) vector treatments (Figure 5), but not in the retinas receiving control vector containing the coding sequence for secreted GFP without Ang-(1-7) or ACE2 (data not shown).

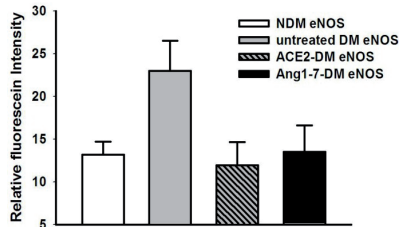


Figure 5. Effects of ocular treatments with ACE2 and Ang(1-7)-AAV2 on retinal vascular permeability in diabetic eNOS^{-/-} mice. Retinal vascular permeability was evaluated by FITC-labeled albumin extravasations and quantified by measuring the fluorescence intensity in serial sections from eNOS^{-/-} mice at 1 month after induced diabetes. Data are presented as mean \pm SD from 6 eyes in each group. * $p < 0.01$ (versus untreated DM group). NDM: non-diabetics; DM: diabetes. (From [108] with permission of Mol. Therapy).

3.3.2. Increased expression of ACE2 and Ang1-7 resulted in reduced ocular inflammation in diabetic retina

Diabetes-induced ocular inflammation, as demonstrated by increased infiltrating CD45 positive macrophages and activation of CD11b positive microglial cells, was significantly reduced in eyes treated with ACE2 and Ang-(1-7) expression vectors (Figure 6).

3.3.3. Increased ACE2/Ang1-7 expression reduced the number of acellular capillaries in the diabetic retina

Induction of diabetes for 2 month in eNOS^{-/-} mice resulted in a >10-fold increase in the formation of acellular capillaries that was significantly reduced in diabetic retinas which received ACE2/Ang-(1-7) vector treatments (Figure 7). Furthermore, increasing the level of ACE2 also prevented basement membrane thickening in diabetic eNOS^{-/-} retina (Figure 8).

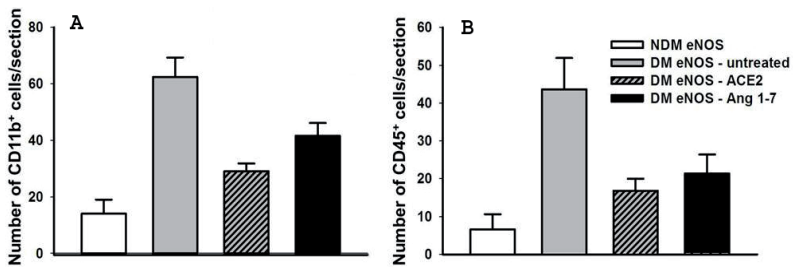


Figure 6. Intravitreal administration of ACE2 or Ang-(1-7)-AAV reduces diabetes-induced ocular inflammation. A. Quantification of CD45positive inflammatory cells in the retinas from untreated non-diabetic, ACE2 treated and Ang-(1-7) treated diabetic eNOS^{-/-} mouse retinas at 1 month after induced diabetes or the equivalent age in untreated controls. B. Quantification of CD11b positive inflammatory cells in the retinas from untreated non-diabetic, ACE2 treated and Ang-(1-7) treated diabetic eNOS^{-/-} mouse retinas at 1 month after induced diabetes or the equivalent age in untreated controls. N=4 for each group. *p<0.01 (versus untreated DM group). (From [108] with permission of Mol. Therapy).

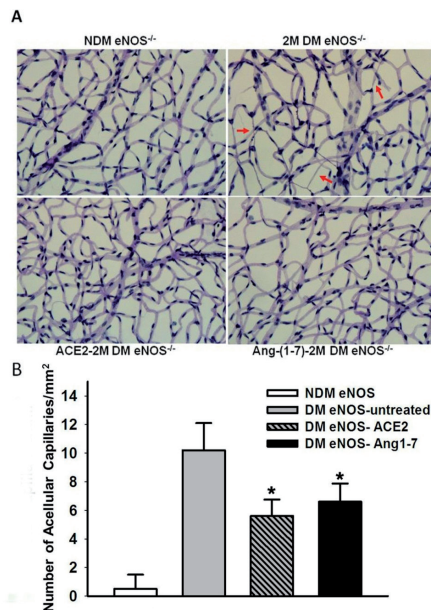


Figure 7. Evaluation of acellular capillary formation in untreated and AAV-ACE2/Ang-(1-7) treated retinas of diabetic mice. Treatments with ACE2 and Ang 1-7 vectors in the diabetic eNOS^{-/-} mouse retinas reduced acellular capillaries. A: Representative images of trypsin-digested retinal vascular preparations from untreated non-diabetic eNOS^{-/-}, ACE2 and Ang-(1-7) treated diabetic eNOS^{-/-} mouse retinas (2 months after induced diabetes or the equivalent age in untreated controls). Arrows indicate the acellular capillaries. B. Quantitative measurements of acellular capillaries. The values on Y-axis represent the number of acellular capillaries per mm² retina. NDM: non-diabetes; DM: diabetes. N=6. *p<0.01 (versus untreated DM group). (From [108] with permission of Mol. Therapy).

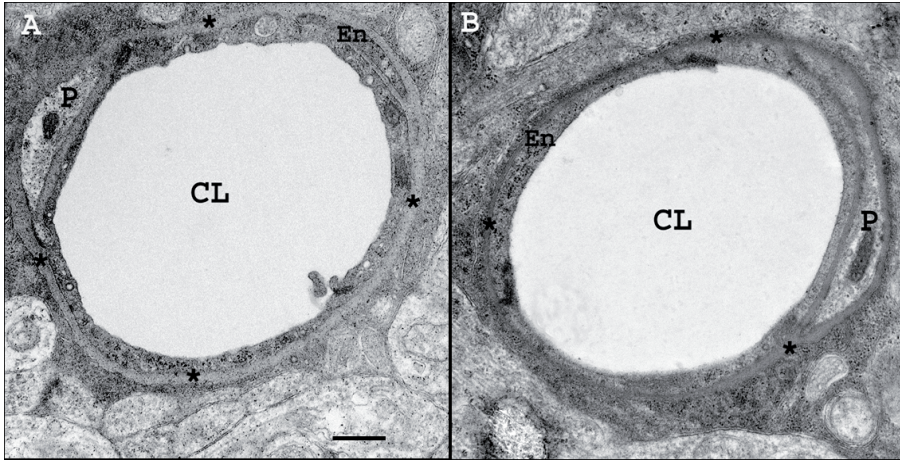


Figure 8. Transmission electron micrographs of retinal capillaries from a untreated 2 month diabetic $eNOS^{-/-}$ mouse eye (A), and an eye that received AAV-ACE2 treatment 2 weeks before STZ-induction of diabetes (B). CL: capillary lumen; En: endothelial cell; P: pericyte; * indicates the capillary basement membrane. Scale bar = 500nm. We have previously shown that the basement membranes of retinal capillaries from the diabetic $eNOS^{-/-}$ animals at two months after STZ induction of diabetes was significantly thicker than those from age-matched, non-diabetic animals [107]. The thickening of the basement membrane was prevented in the AAV-ACE2 treated eyes (73.81±17nm, versus 95.72±20 nm in untreated DM eye).

3.4. Protective role of ACE2/Ang (1-7) AAV gene delivery in a rat model of DR

3.4.1. Increased ACE2/Ang1-7 expression reduced the number of acellular capillaries in the diabetic rat retina

We also used STZ-induced diabetic SD rats as an additional animal model of diabetes to provide conceptual validation. We observed more than a 5-fold increase in the number of acellular capillaries in STZ-induced diabetic rat retinas at 14 month of diabetes. This increase was almost completely prevented by gene delivery of either ACE2 or Ang-(1-7) (Figure 9).

3.4.2. Increased expression of ACE2/Ang-(1-7) reduces oxidative damage in diabetic retina

Diabetes and its complications are associated with increased oxidative stress. We assessed oxidative damage measuring the levels of thiobarbituric acid-reactive substances (TBARs, is a marker for oxidative damage [110]) in the retina). Diabetes induced a significant increase in TBARs (Figure 10A) in $eNOS^{-/-}$ mouse retinas (Figure 10A). This increase is completely prevented by AAV-ACE2 or Ang-(1-7) treatment. Similar results were also obtained in SD rat retinas (Figure 10B).

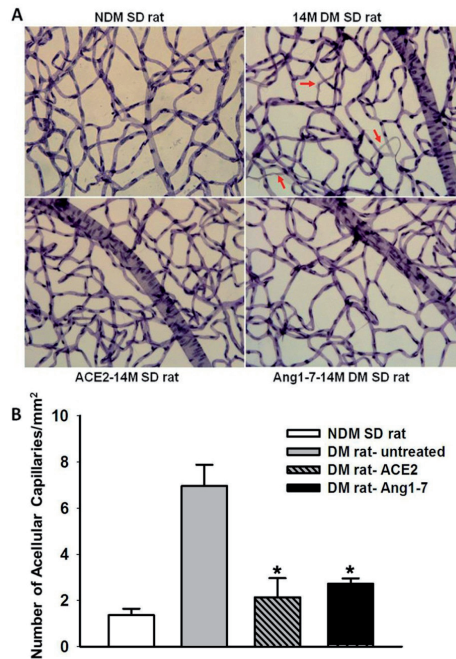


Figure 9. Evaluation of acellular capillary formation in untreated and ACE2/Ang-(1-7) AAV2 vector treated retinas of diabetic SD rats. (A) Representative images of trypsin-digested retinal vascular preparations from non-diabetic SD rat, untreated, ACE2 and Ang-(1-7) treated diabetic SD rat retinas (14 months after induced diabetes). (B) Quantitative measurements of acellular capillaries. Values on Y-axis represent the number of acellular capillaries per mm² of retina. NDM: non-diabetes; DM: diabetes. N=6. *p<0.01 (versus untreated DM group). (From [108] with permission of Mol. Therapy).

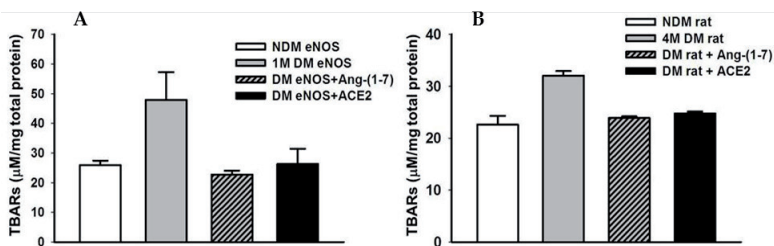


Figure 10. TBARs levels in eNOS^{-/-} mouse retinas (A) and SD rat retinas (B). Diabetes resulted in increased TBARs levels in both eNOS^{-/-} mouse retinas at 1 month of diabetes and SD rat retinas at 4 months of diabetes. These increases were prevented by AAV-ACE2/Ang-(1-7) treatments. NDM: non-diabetes; DM: diabetes. N=6/group. *p<0.01 (vs untreated DM). (From [108] with permission of Mol. Therapy).

3.5. Possible mechanisms of protective action of ACE2/Ang (1-7) in diabetic retina

We demonstrate that all the genes within the RAS are expressed in the retina, consistent with various previous reports (reviewed in [111] and references therein), and the expression levels of genes in the vasoconstrictive arm of RAS (renin, ACE, AT1R) are highly elevated in diabetic retinas, whereas there is initial increase in the expression of genes in the vasodilative axis (ACE2 and MAS) earlier in diabetes that attenuate over time with the progression of diabetes, thus tipping the balance towards more vasoconstrictive, proinflammatory, hypertrophic effects of RAS mediated by ACE/Ang II/AT1R axis. This is associated with increased ACE activity and Ang II levels in diabetic retinas, whereas ACE2 activity and Ang-(1-7) levels are not significantly changed, while the mRNA levels for ACE2 and Mas receptor are reduced under these conditions.

Furthermore, we show that enhanced expression of either ACE2 or Ang-(1-7) via AAV vector mediated gene delivery in the retina prevents diabetes-induced retinal vascular permeability, thickening of basement membrane, retinal inflammation, formation of acellular capillaries, and oxidative damage in both mouse and rat models of diabetic retinopathy. More importantly, these beneficial effects occur in the absence of systemic control of glucose, blood pressure, which is elevated in eNOS^{-/-} mice [107], and other diabetic complications [112], suggesting that local RAS activation plays a significant role of pathogenesis of diabetic retinopathy, and can be modulated locally to restore the balance between the two counter-acting arms by enhancing the ACE2/Ang-(1-7)/MAS axis. These observations provide conceptual support that enhancing ACE2/ Ang-(1-7) axis maybe an effective strategy for the treatment of DR.

Although various components of RAS have been detected in retina, our study is the first to examine the expression levels of all known RAS genes during the progression of diabetes in the eNOS^{-/-} mice, which exhibit accelerated retinopathy [107]. We show that increased expression of genes in the vasoconstrictive, proinflammatory axis of RAS (ACE, AT1R, renin, renin receptor) occur early, 14 days after STZ-induced diabetes. We have previously shown that increased retinal vascular permeability and gliosis are already detectable at this time point in diabetic eNOS^{-/-} mouse retina, suggesting that local hyperactivity of the deleterious axis (ACE/Ang II/AT1R) may contribute to these pathological changes. We also measured ACE and ACE2 activities in diabetic eNOS^{-/-} mouse retina. In contrast to a previous report which showed that ACE enzyme activity was decreased, whereas ACE2 enzyme activity was increased in diabetic rat retinas [113], we found that ACE activity is highly increased in diabetic retinas, whereas ACE2 activity remains unchanged. This discrepancy may be due to the difference in animal models or the time points at which these assays were performed.

The importance of the vasodeleterious axis of the RAS (ACE/ Ang II/ AT1R) in cardiovascular disease, as well as in diabetes and diabetic complications, is well established since ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs) are leading therapeutic strategies [20, 33-35]. However, the impact of the vasoprotective axis of the RAS remains poorly understood, particularly in the eye. The concept that shifting the balance of the RAS towards the vasodilatory axis by activation of ACE2 or its product, Ang-(1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis [24, 36-38]. We

show that increased expression of either ACE2 or Ang-(1-7) is protective in both eNOS^{-/-} mouse and rat models of diabetic retinopathy. However the action of ACE2 and Ang-(1-7) may be different. The protective effect of ACE2 may result from reduced Ang II, by catalyzing its conversion to Ang-(1-7), thus increasing the level of Ang-(1-7), or combination of both. Indeed, in the AAV-ACE2 treated retina diabetes-induced elevation of Ang II is reduced and this is associated with an increased level of Ang-(1-7). On other hand, the fact that increased Ang-(1-7) expressed from AAV vector in the retina is also protective and that the Ang II level remained high in AAV-Ang-(1-7) treated retinas suggest that Ang-(1-7) can produce physiological responses that direct counteract these of Ang II, consistent with well-established effects of Ang-(1-7) [114].

It is interesting to note that ACE2 over-expression resulted in reduced Ang II and increased Ang-(1-7) levels as expected, but has no effect on ACE activity. However, over-expression of Ang-(1-7) had no effect on endogenous ACE2 activity, but significantly reduced ACE activity. Paradoxically, despite reduced ACE activity in AAV-Ang-(1-7) treated retinas, Ang II levels remained high. It is possible that other enzymes/pathways may be involved in Ang II formation in addition to ACE. One such candidate is chymase, which has been detected in vascular systems and other tissues including eye [115]. Another candidate is the receptor for prorenin and renin (pro/renin). It has been recently demonstrated that binding of pro/renin to its receptor, pro/renin receptor (PRR), causes its prosegment to unfold, thereby activating prorenin so that it is able to generate angiotensin peptides that stimulate the Ang II-dependent pathway [76]. Considering the fact that retina contains high level of prorenin, and its level is further increased in patients with diabetic retinopathy [52], this pathway likely contributes to increased Ang II level under diabetic conditions. The existence of multiple pathways for Ang II formation at the tissue level may explain the limited beneficial effects of classic RAS blockers, and may also lend support for the notion that enhancing the protective axis of RAS (ACE2/Ang-(1-7)/Mas) may represent a more effective strategy for treatment of diabetic retinopathy and other diabetic complications.

AAV vector mediated gene therapy for ocular diseases has been studied in animal models for more than a decade. Reports focusing on retinal therapy include a wide variety of retinal degenerative animal models of corresponding human retinopathies, as well as the therapeutic effects of AAV-vector mediated expression of neuroprotective, anti-apoptotic, and anti-angiogenic agents in the retina [116]. In view of recent clinical trials in which AAV delivered RPE65 gene led to restoration of vision in human patients and other reports on successful trials on treatment of ocular diseases and inherited immune deficiencies (reviewed in [117] and references therein), gene therapy has emerged as promising approach and may become a standard treatment option for a wide range of diseases in the future. In particular, when considering that the diabetic individual experience this serious ocular complication for decades, a therapeutic strategy that is long-lasting and does not require patient compliance is particularly desirable. Thus, the delivery of ACE2 and/or Ang-(1-7) could serve as a novel gene therapeutic target for DR in combination with existing strategies to control hyperglycemic and insulin resistance states.

4. Summary

All genes of the RAS are locally expressed in the retina, establishing the existence of an intrinsic retinal RAS. It is clear that the expression of genes of the vasoconstrictive/pro-inflammatory/ proliferative/fibrotic (i.e., vasodeleterious) axis (ACE/Ang II/AT1R) is highly elevated, while the vasoprotective axis [ACE2/Ang-(1-7)/Mas] is decreased in the diabetic retina. We have demonstrated that increased expression of ACE2 or Ang-(1-7), two key members of the vasoprotective axis, via AAV-mediated gene delivery to the retina attenuates diabetes-induced retinal vascular pathology. Moreover, these beneficial effects of gene transfer occur without influencing the systemic hyperglycemic status. Thus, strategies enhancing the protective ACE2/Ang-(1-7) axis of RAS could serve as a novel therapeutic target for DR.

5. Implications and future challenges

Hyperactivity of RAS, resulting in elevated concentrations of the principal effector peptide Ang II, is central to pathways leading to increased vascular inflammation, oxidative stress, endothelial dysfunction and tissue remodeling in variety of conditions including heart failure, stroke, renal failure, diabetes and its associated complications including DR. As a result, RAS inhibitors are one of the first-line therapeutic agents for treating patients with cardiovascular diseases, metabolic syndrome, diabetes and diabetic complications. Ang II blockade has shown to be antiangiogenic [66, 118, 119], anti-inflammatory [120] and improves retinal function [65], and indeed Ang II blockade therapy for retinopathy is in several clinical trials [67, 68, 121]. Despite the clear beneficial effects of RAS blockers (ACE inhibitors [ACEi] and angiotensin receptor blockers [ARBs]) [70, 71, 122], end-organ damage still ensue in patients with diabetes. Overwhelming evidence now supports the notion that activation of RAS at tissue levels contributes to the development and progression of diabetic complications including DR, independent of circulating RAS regulation. However the precise molecular and cellular mechanisms as to how retinal RAS contributes to the development and progression of DR remain to be elucidated. Recent studies have also revealed the evolving complexity of RAS with a myriad cellular and intracellular pathways leading to formation of Ang II, as well as Ang II- independent signaling pathways resulting in hyperactivity of tissue RAS. The physiological implications of many of these components are still not well understood and new antagonists/agonists specific to these new components remain to be discovered. Nevertheless, our results clearly demonstrate that enhancing the protective axis of RAS (ACE2/Ang1-7/Mas) locally may be a better strategy for counteracting the effects of the pathological RAS activation than present systemic approaches. Furthermore, since AAV vector mediated gene delivery has been shown to be safe, and improve vision for extended periods of time after a single administration in several clinical trials, enhancing the endogenous protective axis of RAS (ACE2/Ang1-7/Mas) by local gene delivery, in combination with combination with existing strategies to control hyperglycemic and insulin resist-

ance states may represent a better strategy for preventing and treating diabetic complications such as diabetic retinopathy.

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Gene Therapy for Retinitis Pigmentosa

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Additional information is available at the end of the chapter

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1. Introduction

The retina comprises diverse differentiated neurons that have specific functions. Photoreceptor cells, the first-order neurons in the retina, have photopigments (rhodopsin and opsin) that absorb photons. Signals produced by the photoreceptor cells are transmitted to second-order neurons. Finally, visual signals are transmitted to the brain from the third-order neurons, the retinal ganglion cells (RGCs). Major diseases that cause blindness in advanced countries include glaucoma, diabetic retinopathy, retinitis pigmentosa (RP), and age-related retinopathy. Loss of vision due to these diseases is irreversible. However, with regard to glaucoma, eye drops that have the effect of reducing intraocular pressure have been developed. In diabetic retinopathy, effective surgical treatments such as vitrectomy and photocoagulation have been established. Blindness due to glaucoma and diabetic retinopathy can be prevented by administering these treatments in the early phase. On the other hand, in diseases caused by gene mutations, such as RP, effective treatments for delaying photoreceptor degeneration have not yet been established. Degeneration of photoreceptor cells results in loss of vision, even if other retinal neurons are intact [1-3].

RP is a disease that causes blindness due to photoreceptor degeneration. Symptoms include night blindness and loss of peripheral and central vision. Approximately 1 in 4,000 people are affected by this disease [4]. In 1990, Dryja et al. [5] first identified a point mutation in the rhodopsin gene from RP patients. A number of gene mutations responsible for RP has subsequently been identified. Most of these genes are associated with the phototransduction pathway in the retina. In some cases, the mutated gene exists not only in photoreceptor cells but also in retinal pigment epithelial cells. To date, 53 causative genes and 7 loci of RP have been identified (<http://www.sph.uth.tmc.edu/Retnet/>). Leber's congenital amaurosis (LCA) is another retinal degenerative disease predicted to affect approximately 1/81000 individuals [6]. Most LCA patients have

severe visual defects in childhood. Histological analysis of the retinas of LCA patients shows marked retinal atrophy in the outer retinal layer, vascular thickening and sclerosis, and atrophy of the retinal pigment epithelium (RPE) [7]. Leber classified the disease as a type of RP on the basis of these characteristics. Later, Franceschetti and Dieterle differentiated it from retinal dystrophy based on the features of electroretinograms (ERGs) in these patients. Many gene mutations involved in LCA have been identified and the disease has been classified into 15 subtypes based on the affected gene [8-13]. Among these, LCA2, accounting for 10% of LCA cases [14], is due to a mutation in the RPE65 gene, which encodes all-*trans* retinyl ester isomerase. Deficiency in RPE65, leads to severe loss of visual function. Thus, in the case of LCA2, the cause of the disease is clearly identified as the biochemical blockade of the visual cycle caused by RPE65 deficiency [11,12]. Replacement therapy using the RPE65 gene is a candidate therapeutic strategy for LCA2. Indeed, successful results have been reported in RPE65 replacement therapy with the LCA2 animal model, Briard dogs [15]. After proof-of-principle studies [16], phase I trials using adeno-associated virus vector type 2 were conducted in 3 independent groups [17]. The results showed no adverse effects such as systemic dissemination of vector or immunological responses to the vector or transgene. Importantly, improvement of visual function as evaluated by microperimetry was observed in 1 subject [18,19]. Two other groups also reported improvement in visual function [20,21]. Continuous follow-ups for 1.5 years [22] have confirmed the safety and tolerability of replacement gene therapy [23]. The various hereditary forms of RP are as follows: autosomal dominant, recessive, and X-linked recessive. The Pro23->His gene mutation in the rhodopsin gene [24,25] occurs in 20–30% of all RP patients in Europe and the U.S. In contrast, the occurrence in Japan is only a few percent. Thus, in addition to the diversity of the gene mutations, their frequencies vary characteristically among different races. Differences in the progression, clinical findings, and development of the disease are also observed among different patients, even in those with the same mutation. A common feature of photoreceptor cell death caused by various gene mutations is eventual apoptosis via a common pathway [26]. Based on this rationale, various kinds of methods to prevent apoptosis, such as chemical treatment [27,28] and gene therapy, including gene replacement and neurotrophic factor supplementation [29-31], have been investigated. However, these strategies have not been successful in the complete prevention of cell death, although they have been shown to delay degeneration. The diversity of clinical features and gene mutations makes it difficult to develop effective treatments for RP.

A retinal prosthesis, comprising electrodes, an image processor, and a camera, is the only method to restore vision that has been studied [32-36]. Recently, a new strategy involving gene therapy for restoring vision has been developed using bacteriorhodopsin family genes [37,38]. The channelrhodopsin-2 (ChR2) gene derived from the green alga *Chlamydomonas* functions as a photoreceptor and cation-selective channel [39]. After the absorption of photons by photopigments, photon acquisition is completed by a chain reaction involving certain photoreceptor-specific proteins. Thus, the phototransduction pathway in photoreceptor cells requires not only photopigments but also certain photoreceptor-specific proteins, which complicates the reaction. Due to the inherent characteristics of ChR2, photosensitive neurons can be produced by the transfer of the ChR2 gene into neurons [40-42]. Here, we introduce new strategies for restoring vision by using channelrhodopsins.

2. Materials and methods

All the experiments performed for this report were approved by the Tohoku University Animal Care Committee, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number and suffering of animals used in the following experiments.

Animals

We used 2 types of photoreceptor degeneration models: a genetically blind rat model and a light-induced photoreceptor degeneration model. The experimental design for each of these models is shown in Fig. 1.

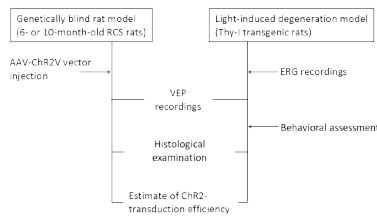


Figure 1. Experimental design. Two types of photoreceptor degeneration models were used in this study. The photoreceptor cells of RCS rats degenerate by 3 months after birth due to the *Mertk* gene mutation. On the other hand, Thy-TG rats have native photoreceptors. Therefore, we subjected TG rats to continuous light exposure to induce photoreceptor degeneration. To confirm photoreceptor degeneration, ERGs were recorded before performing behavioral assessments. Finally, the eyes from all animals were subjected to histological examination.

Genetically blind rats

Royal College of Surgeons (RCS; rdy/rdy) rats [43,44] were used as model animals for photoreceptor degeneration in our experiments. The RCS rat, an animal model of recessively inherited retinal degeneration, is widely used in the study of photoreceptor degeneration. The gene responsible is the receptor tyrosine kinase gene *Mertk* [45], and mutations in *MERTK*, the human ortholog of the RCS rat retinal dystrophy gene, cause RP [46]. Photoreceptor degeneration is almost complete by 3 months after birth. We intravitreally injected the AAV-ChR2V vector into 6-month- or 10-month-old RCS rats. The rats were obtained from CLEA Japan, Inc. (Tokyo, Japan).

Thy-1 ChR2 transgenic rats

We established transgenic (TG) rats harboring the ChR2 gene regulated by the Thy-1.2 promoter to investigate contrast sensitivity at each spatial frequency [47]. The rat Thy-1.2 antigen has been found to be abundant in the brain and thymus [48,49]. In the retina, the Thy-1.2 antigen is recognized as a marker specific to RGCs [50,51]. It is necessary to induce the degeneration of native photoreceptor cells in order to investigate the visual function conferred by ChR2-expressing RGCs, because the Thy-1 TG rat has native photoreceptor cells. For this purpose, Thy-1 TG rats were subjected to light-induced photoreceptor degeneration. Briefly, Thy-1 TG rats were kept in cyclic light (12 hours ON/OFF: 5–10 lux/dark) for at least 2 weeks

prior to light exposure. The rats were then exposed to a 3000-lux intensity of fluorescent light for 7 days [28]. We used a light exposure box (NK Systems, Tokyo, Japan) to control the timing and light intensity for the induction of photoreceptor degeneration. After induction, we recorded ERGs to confirm photoreceptor degeneration.

Preparation of the adeno-associated virus vector

The adeno-associated virus (AAV) vector with the ChR2 gene was constructed as described previously [38]. Following this, the AAV Helper-Free System (Stratagene, La Jolla, CA) was used to produce infectious AAV-Venus (control) and AAV-ChR2V virions, which were purified by a single-step column purification method as previously described [52].

Recording of ERGs and visual electrophysiology (VEP)

ERGs and VEP readings were recorded using a Neuropack (MEB-9102; Nihon Kohden, Tokyo, Japan) according to methods previously described [38,53]. Briefly, rats were dark-adapted overnight, and the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Small contact lenses with gold wire loops were placed on both corneas, and a silver wire reference electrode was inserted subcutaneously between the eyes. Eyes were stimulated with flash light stimuli of 10-ms duration using a blue LED. Full-field scotopic ERGs were recorded, band-pass filtered at 0.3–500 Hz, and averaged for 5 responses at each light intensity. For VEP recordings, recording electrodes (silver-silver chloride) were placed epidurally on each side, 7 mm behind the bregma and 3 mm lateral of the midline, and a reference electrode was placed epidurally on the midline 12 mm behind the bregma, at least 7 days before the experiments [54,55]. Under ketamine-xylazine anesthesia, the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. The ground electrode clip was placed on the tail. Photic stimuli of 20-ms duration were generated under various intensities by pulse activation of a blue LED. The high- and low-pass filters were set to 50 kHz and 0.05 kHz, respectively. One hundred consecutive response waveforms were averaged for each VEP measurement.

Determination of transduction efficiency

At the end of the experiment, RCS and Thy-TG rats were sacrificed, and their eyes were resected and fixed in 4% paraformaldehyde and 0.1 M phosphate buffer, pH 7.4 [56]. The eye of each rat was flat-mounted on a slide and covered with Vectashield medium (Vector Laboratories, Burlingame, CA) to prevent the degradation of fluorescence. Then, the number of positive cells was counted.

3. Behavioral assessment

The spatial vision of each animal was quantified by its optomotor response. We used a virtual optomotor system to evaluate the contrast sensitivities of each spatial frequency. The original virtual optomotor system described by Prusky et al. [57] was modified for rats [47]. When a drum is rotated around an animal with printed visual stimuli on the inside wall, the animal tracks the stimulus by turning its head. A light-dark grating pattern was displayed on

computer monitors (ProLite E1902WS; Iiyama, Tokyo, Japan) arranged in a square around a platform. The software controlled the speed of virtual optomotor rotation, which was set at 12 degrees per second (2 rpm) in all experiments. The spatial frequency and the contrast of the grating pattern were varied but the average brightness was kept constant.

The animal was allowed to move freely on the platform in the virtual optomotor system. The grating session was started at a low spatial frequency (0.06 cycles/degree) with maximal contrast. An experimenter assessed whether the animals tracked the rotation, by monitoring the head movement and the presented rotating stimulus simultaneously on another display connected to the video camera. If head movement simultaneous with the rotation was evident, the experimenter judged that the animal could discriminate the grating, and proceeded to the next grating session. If the movement was ambiguous, the same grating session was presented again. All behavioral tests were double-blinded and performed during the first few hours of the animals' light cycle (light on at 8 AM).

4. Results

4.1. Recording of VEP measurements in RCS rats

VEP measurements in 6- or 10-month-old RCS rats are expected to be abolished due to loss of photoreceptor cells. Generally, in RCS rats, photoreceptor degeneration is almost complete by 3 months after birth. Indeed, VEP measurements were not evoked even by the maximal LED flash in any of the aged RCS (rdy/rdy) rats (Fig. 2A). On the other hand, robust VEPs were evoked by the blue LED flash in RCS rats injected with the AAV-ChR2V vector (Fig. 2A). Initially, small VEP responses were observed at 2 weeks after AAV injection (data not shown), and the maximum amplitudes of VEP were observed 8 weeks later [58]. There were notable differences in sample waveforms from 6- and 10-month-old rats injected with AAV-ChR2V. Amplitudes and latencies of VEPs from 6-month-old rats were larger and shorter, respectively, than those from 10-month-old rats (Fig. 2B).

4.2. Transduction efficiencies of ChR2 in retinas of RCS rats

The expression of the ChR2 gene was evaluated by measuring Venus fluorescence in RCS rat retinas (Fig. 3A). The number of positive cells in rats injected at 10 months of age was significantly less than that injected at 6 months of age (Fig. 3B). The number of RGCs decreased linearly with age, following photoreceptor degeneration in the RCS rats (Fig. 3C). We have previously shown [56] that the ChR2 gene is mainly expressed in RGCs upon intravitreal injection of the AAV-ChR2V vector. Therefore, the observed decrease in the number of RGCs with age suggests that the transduction efficiencies at both ages are very similar.

4.3. Photoreceptor degeneration in Thy-1 TG rats

There were 11–12 rows of photoreceptor nuclei in the outer nuclear layer (ONL) of the Thy-1 TG rats; this is a number usually observed in rodents without retinal degeneration [59].

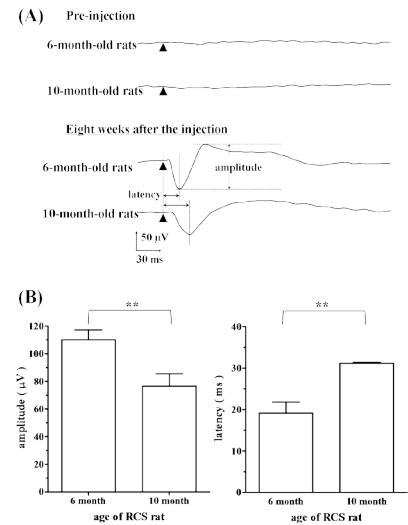


Figure 2. VEP recordings before and after the injection of AAV-ChR2V. (A) VEP recordings from both 6-month- and 10-month-old RCS rats showed no responses. However, VEPs responses were clearly elicited 8 weeks after injection. (B) The amplitudes and latencies from rats injected with AAV-ChR2 at 6 months of age ($n = 8$) were significantly larger and shorter than those injected at 10 months of age ($n = 4$).

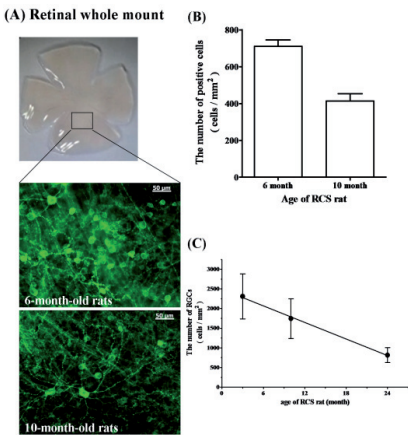


Figure 3. Transduction efficiencies of ChR2 in retinas of RCS rats. (A) Retinal whole-mount specimens obtained from rats injected with AAV-ChR2 at 6 and 10 months of age. (B) Venus-positive cells expressing the ChR2 gene were observed in whole-mount specimens. (C) The number of RGCs decreased with age.

Following continuous light exposure, photoreceptor cells disappeared (Fig. 4A). ERGs showed no response, indicating that the photoreceptor cells degenerated in the whole retina (Fig. 4B).

However, robust VEP measurements could be recorded, even though the photoreceptor cells had completely degenerated (Fig. 4B). Intense expression of the ChR2 gene was observed in the entire retina, with about 45% of RGCs positive for ChR2 (Fig. 4C).

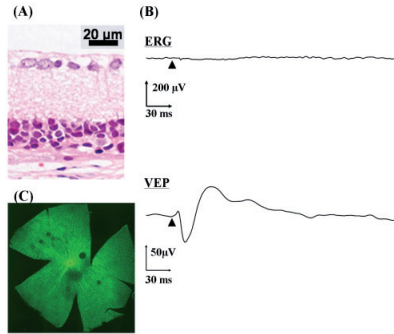


Figure 4. Electrophysiological response of Thy-I TG rats after photoreceptor degeneration. (A) Hematoxylin-eosin staining of the retina showed the degeneration of the native photoreceptor cells after continuous light exposure. (B) Extensive expression of the ChR2 gene was observed throughout the retina. (C) The ERG response was completely abolished following continuous light exposure, indicating that native photoreceptor cells had degenerated throughout the retina. VEP measurements could still be recorded after photoreceptor degeneration.

4.4. Behavioral assessment in photoreceptor degenerated-Thy-I TG rat

In our virtual optomotor system, a stimulus of blue stripes over a black background was produced according to a sine wave function with variable amplitude and frequency (Fig. 5A). All the photoreceptor-degenerated Thy-I TG and wild-type (normal) rats tracked the virtual rotating blue/black gratings (Fig. 5B). However, tracking stopped when the contrast was reduced below a specific threshold. We observed that contrast sensitivity was small at the minimal spatial frequency of 0.06 cycles per degree (CPD), increased with an increase in spatial frequency, and was negligible at spatial frequencies over 0.52 CPD. Therefore, the relationship followed an inverted U-shaped curve, as noted in previous reports [57]. In photoreceptor-degenerated Thy-I TG rats, no reduction of contrast sensitivity was observed at any spatial frequency. Unexpectedly, the contrast sensitivity was instead somewhat enhanced at low spatial frequencies such as 0.09 or 0.18 CPD (Fig. 5C).

5. Discussion

The photo-acquisition system of mammalian photoreceptor cells, which mediates various photoreceptor-specific proteins, is very complicated. In contrast, the corresponding system in green algae such as *Chlamydomonas* and *Volvox* is simpler. ChR2 contains a 13-*cis* retinal that absorbs a photon, inducing a conformational change. The ChR2 functions as a cation-selective ion channel. For this reason, the transfer of a single gene, ChR2, to RGCs allows the generation

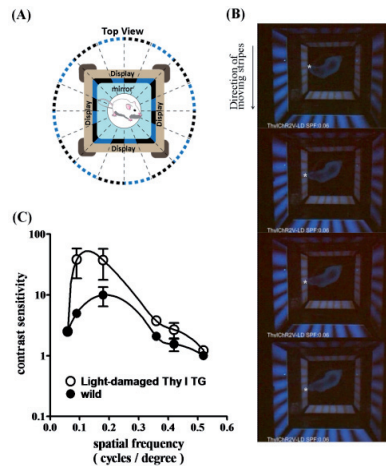


Figure 5. Behavioral assessment using a digital optomotor. (A) The digital optomotor consisted of 4 displays surrounding a platform. The number of stripes and the contrast were controlled by software. (B) Superimposed images from movies showed that the rats were able to discriminate the moving stripes. The arrow indicates the direction of the moving stripes. The asterisk indicates the point of the rat's nose. (C) The contrast sensitivities of photoreceptor-degenerated Thy-1 TG rats were higher at low spatial frequencies compared to those of wild-type (normal) rats ($n = 8$).

of photosensitive RGCs. In the normal visual pathway, the light incident upon the eyes is first received by photoreceptor cells located at the end of the retinal layers. The photoreceptor cells control neurotransmitter release, and second-order neurons located in the inner nuclear layer respond to the neurotransmitter. Finally, RGCs produce action potentials and transmit to the lateral geniculate nucleus (LGN) via the optic nerve (Fig. 6). In RP, the photo-acquisition system is damaged due to the degeneration of photoreceptor cells, even if the other retinal layers remain intact. RGCs that are rendered photosensitive by the transfer of the ChR2 gene can directly respond to light and transmit signals to the brain. In this newly organized photo-acquisition system, the other retinal neurons besides the RGCs are not required for the perception of light.

Although VEP responses recovered after ChR2 gene transfer, the amplitudes and waveforms were different between rats injected with AAV-ChR2V at 6 and 10 months of age. One possibility is that RGC activity decayed after photoreceptor degeneration. However, our data show that the number of RGCs decreased after photoreceptor degeneration (Fig. 3C). The calculated RGC transduction efficiencies in 6-month-old rats were the same as those in 10-month-old rats. The differences in the recorded amplitudes and latencies shown in Fig. 2 appear to be due to differences in the number of photosensitive RGCs. We previously reported that the RGC transduction efficiency in 10-month-old rats was about 28% [38]. Subsequently, Isago et al. showed that the RGC transduction efficiencies in 6- and 10-month-old rats were 28.3 and 27.7%, respectively [56]. The data clearly indicates that the transduction efficiency is the same, although the number of ChR2-expressing cells was lower, corresponding to the decrease in the number of RGCs.

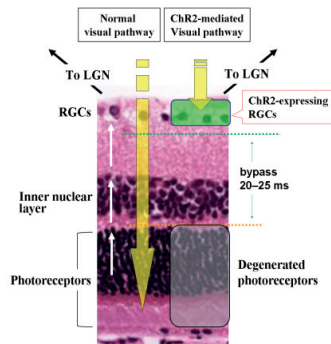


Figure 6. Summary of the visual pathway in ChR2-expressing RGCs. In the normal visual pathway, light (visual signals) is received by photoreceptor cells located at the end of the retinal layer. Photoreceptor cells produce signals that are transmitted to the inner nuclear layer. Finally, RGCs generate action potentials and thereby transmit signals to the LGN. RGCs play a role in transferring the visual signal to the brain. On the other hand, ChR2-expressing retinal ganglion cells directly receive light, produce action potentials, and transmit them to the LGN. Therefore, there is no need for mediation by other retinal cells.

To investigate visual acuity resulting from ChR2-expressing RGCs, we established a TG rat model expressing the ChR2 gene in RGCs. Photoreceptor-degenerated TG rats clearly tracked the rotation of blue-black stripes in a virtual optomotor. However, RCS rats that received the ChR2 gene in the AAV vector did not track the rotation of the virtual optomotor at any spatial frequency. Recently, we tested the behavior of RCS rats using a mechanical optomotor system and showed that the intensity of luminosity the rat received was the most important factor influencing their tracking of the rotation of the column [53]. A luminosity of over 500 lux was needed to induce head tracking in ChR2-expressing RCS rats. However, the maximum luminosity of the virtual optomotor was about 100 lux. It was therefore too low to induce head tracking in RCS rats. The question then arises: what is the difference between the TG and RCS rats? We do not have a reasonable explanation for this. One possibility is that the number of ChR2-expressing RGCs in the TG rat is greater than that in the RCS rat. About 45% of the RGCs expressed ChR2 in the TG rat. Compared to the TG rat, the transduction efficiency in the RCS rat is about 28% independent of the age of the animal. This may affect the light sensitivity. As the another explanation, in the case of TG rats, ChR2 is expressed after birth; therefore, there is a possibility that retinal organization and function might be altered, that cannot be ruled out.

RGCs are merely one of the candidate cell types that could receive the ChR2 gene. Lagali et al. [60] succeeded in transferring the ChR2 gene into ON-bipolar cells in the retina and confirmed the restoration of visual and behavioral responses. ON- and OFF-bipolar cells receive synaptic input from photoreceptors. Considering that ChR2 can elicit light-on responses, ON-bipolar cells seem to be the most appropriate cells for the transfer of the ChR2 gene. However, 2 questions arise in this regard. First, how can we deliver the ChR2 gene into ON-bipolar cells for human gene therapy? Lagali et al. [60] transferred the ChR2 gene into neonatal mice by electroporation of the plasmid vector. It is generally difficult to transfer a gene into the depths

of the retina via intravitreal injection of AAV vectors, in spite of the development of various serotypes of AAV vectors for retinal gene therapy [61-65]. Second, does synaptic transmission remain intact after photoreceptor degeneration? Some studies have reported that retinal remodeling is triggered in bipolar cells and horizontal cells following photoreceptor degeneration [66-70]. Recently, Doroudchi et al. [71] succeeded in transferring the ChR2 gene into ON-bipolar cells by the subretinal injection of a modified AAV vector (AAV8-Y733F) [72] that included a specific promoter for ON-bipolar cells (mGRM6-SV40), and demonstrated the behavioral recovery of the light response. These 2 questions could be resolved by these attractive methods used the specific promoter and the modified AAV vectors if the recovered visual acuity is investigated using a behavioral approach.

Since the discovery of ChR2, bacteriorhodopsins that have similar functions as that of ChR2 derived from *Chlamydomonas* have been identified. Channelrhodopsin-1 from the green alga *Volvox* [73] is a light-activated cation channel that has a different wavelength sensitivity from that of *Chlamydomonas*-derived ChR2. Halorhodopsin, which functions as a light-activated chloride channel, has been identified in *Halobacterium salinarum* [74,75]. Researchers have attempted to discover new light-activated ion channel genes, or to artificially design more functional ones [76-78]. In the future, more effective gene therapy strategies for restoring vision in RP might be developed using newly developed genes and vectors.

6. Conclusion

Target diseases for gene therapy were previously restricted to lethal and severe diseases that lead to death. In our country (Japan), the gene therapy guidelines were updated in 2002, whereby diseases in which bodily functions are severely impaired, such as loss of arms or legs, blindness, and deafness, were added to the list of target diseases for gene therapy. Based on these guidelines, people suffering from impaired vision caused by RP are eligible for gene therapy. However, gene therapy using genes derived from living organisms other than humans has not previously been tested in clinical trials. Safety studies, especially immunological reactions, using appropriate animal models in ChR2-based gene therapy is important before proceeding to clinical trials.

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Gene Therapy for Erythroid Metabolic Inherited Diseases

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Additional information is available at the end of the chapter

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1. Introduction

Gene therapy is becoming a powerful tool to treat genetic diseases. Clinical trials performed during last two decades have demonstrated its usefulness in the treatment of several genetic diseases [1] but also the need to improve vector delivery, expression and safety [2]. New vectors should reduce genotoxicity (genomic alteration due to vector integration), immunogenicity (immune response to gene delivery vectors and/or transgenes) and cytotoxicity (induced by ectopic expression and/or overexpression of the transgene).

In mature erythrocytes, most metabolic needs are covered by glycolysis, oxidative pentose phosphate pathway and glutathione cycle. Hereditary enzyme deficiencies of all these pathways have been identified, being most of them associated with chronic non-spherocytic hemolytic anemia (CNSHA). Hereditary hemolytic anemia exhibits a high molecular heterogeneity with a wide number of different mutations involved in the structural genes of nearly all affected enzymes. Deficiency in metabolic enzymes impairs energy balance in the erythrocytes, with or without changes in oxygen affinity of hemoglobin and delivery to the tissues. Despite of having a better understanding of their molecular basis, definitive curative therapy for Red Blood Cells (RBC) enzyme defects still remains undeveloped.

Conventional bone marrow transplantation allows the generation of donor-derived functional hematopoietic cells of all lineages in the host, and represents the standard of care or at least a valid therapeutic option for many inherited diseases [3]. However, complications associated to allogeneic transplantation can be as severe as the enzymatic deficiency. The recessive inheriting trait of most of these metabolic diseases and the confined enzymatic defect to the hematopoietic/

erythropoietic system, make them suitable diseases to be treated by gene therapy. Correction by gene therapy requires the stable transfer of a functional gene into the autologous self-renewing Hematopoietic stem cells (HSCs) and their mature progeny. Autologous BM transplantation of genetically corrected cells shows several advantages over the allogeneic procedure. First, it overcomes the limitation of human leukocyte antigen (HLA)-compatible donor availability, so it can be applied to every patient. Second, the reduction of morbidity and mortality associated with the transplant procedure, as there is no risk of graft versus host disease (GvHD) and consequently no need for post-transplant immunosuppression.

To date, gene therapy approaches for the treatment of inherited metabolic deficiencies are still limited, mainly because of the frequent lack of selective advantage of genetically corrected cells. This implies that high levels of transgene expression are required, as well as an efficient transduction of HSCs. This requirement have already been described in different RBC diseases as in the erythropoietic protoporphyria (EPP) [4] caused by the deficiency of the last enzyme of the heme biosynthesis pathway or in the piruvate kinase deficiency (PKD) [3], where there is an impairment in the final yield of ATP in RBC. Additionally, some RBC pathologies require switching on expression of the transgene at only the proper stage of differentiation, which represents another challenge in the development of new gene therapy protocols.

2. Gene therapy attempts for inherited metabolic diseases of erythrocytes

Although more than 14 metabolic deficiencies have been identified causing CNSHA, approaches of gene therapy have been done only in a few of them (Table 1). Below, we are including a short description of the different diseases and the attempts addressed.

Among glycolytic defects causing CNSHA, Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common genetic disease. More than 400 million people are affected world wide, showing a vast variability of clinical features. G6PD catalyzes the first reaction of the pentose phosphate pathway, in which Glucose 6-phosphate (G6P) is oxidized and Nicotinamide adenine dinucleotide phosphate is reduced (NADPH) resulting in decarboxylation of CO₂ and pentose phosphate. G6PD plays a central role in the cellular physiology as it is the major source of NADPH, required by many essential cellular systems including the antioxidant pathways, nitric oxide synthase, NADPH oxidase, cytochrome p450 system and others. Indeed, G6PD is essential for cell survival. *G6PD* is a 20 kb X-linked gene that maps to the Xq28 region, consisting of 13 exons and 12 introns, which encode a 514 amino acids protein with ubiquitous expression. More than 100 missense mutations in the *G6PD* gene have been identified [14], being most of them single-point mutations causing an amino acid substitution. Frequently, these mutations cause mild symptoms or no disease, except when patients are challenged by increased oxidative stress or fava beans. However, some mutations provoke severe instability of the G6PD and, as a result, lifelong CNSHA with a variable severity [15,16]. Through genetic studies it has been observed that severe clinical manifestations appear preferentially in exons 7, 10 and 11. As *G6PD* is X-linked, the defect is fully expressed in affected males (hemizygotes who inherit the mutation only from the

mother), whereas in homozygous females the mutations are transmitted from both parents. Thereby, female heterozygotes represent a red blood cell mosaic population, causing a wide range clinical picture.

Disease	Gene	Chrom.	Inheritance	Other symptoms	Bone Marrow Transplantation	Gene Therapy
Glucose-6 Phosphate Dehydrogenase Deficiency (G6PD)	<i>G6PD</i>	Xq28	X-linked	jaundice, spleno- and hepatomegaly, hemoglobinuria, leukocyte disfunction, and susceptibility to infections		D: C57BL/6 mice P: Transduction of 5-FU treated BM cells with MMLV-hG6PD or MPSV-hG6PD vectors and subsequent transplantation. R: lethally irradiated C57BL/6 mice [5]
Pyruvate Kinase Deficiency (PKD)	<i>PKLR</i>	1q21	A.R	Reticulocytosis, splenomegaly, <i>hidrops foetalis</i> , and death in neonatal period	D: normal CBA/N ^{+/+} mice + 5FU R: CBA Pk-1 ^{sc} / PK-1 ^{sc} mice C: minimal (100 or 400 cGy) [6]	D: WT mice P: Transduction of 5-FU treated BM cells with pMNSM-hLPK retroviral vector and subsequent transplantation R: lethally irradiated mice [7]
					D: normal CBA/N ^{+/+} mice R: CBA Pk-1 ^{sc} / PK-1 ^{sc} mice C: no conditioning [8]	D: CBA Pk-1 ^{sc} /PK-1 ^{sc} mice P: Transgenic rescue using the μ LCR-PKLR-hRPK construct [9]
					D: normal Basenji dogs R: PKD Basenji dogs C: sublethal dose (200 cGy) + mycophenolate memofetil + cyclosporine [10]	D: WT mice P: Transduction of Lin Sca1 ⁺ BM cells with a MSFV-hRPK retroviral vector and subsequent transplantation R: lethally irradiated WT mice [11]
					D: HLA-identical sister R: PKD severe patient C: busulfan + cyclophosphamide [12]	D: AcB55 mice P: Transduction of Lin Sca1 ⁺ BM cells with a MSFV-hRPK retroviral vector and subsequent transplantation R: lethally irradiated AcB55 mice [13]
Glucose Phosphate Isomerase Deficiency (GPI)	<i>GPI</i>	19q13.1	A.R	neuromuscular disturbances		
Triose Phosphate Isomerase Deficiency (TPI)	<i>TPI1</i>	12p13	A.R	neuromuscular disorders, mental retardation, frequent infections and death in <i>utero</i>		
Hexokinase Deficiency (HK)	<i>HK1</i>	10q22	A.R	defects in platelets		
Phosphofructokinase Deficiency (PFK)	<i>PFKL</i>	21q22.3	A.R	myopathy, storage disease type VII		
Bisphosphoglycerate Mutase Deficiency (BPGM)	<i>BPGM</i>	7q31-q34	A.R	erythrocytosis		
Glutathion Synthetase Deficiency (GSD)	<i>GSS</i>	20q11.2	A.R	5-oxoprolinuria, metabolic acidosis, central nervous system impairment		

A.R, autosomic recessive; D, donor; R, receptor; C, conditioning; P, protocol

Table 1. Most Common Erythroid Metabolic Inherited Diseases. BM transplantation and gene therapy approaches

Patients with CNSHA suffer anemia and jaundice, but often tolerate their condition well. However, G6PD variants with low activity are related with alterations in the erythrocyte membrane facilitating its breakdown and causing intravascular hemolysis. These symptoms are often accompanied by spleno- and hepatomegaly and hemoglobinuria. Besides, leukocyte dysfunctions caused by lower concentration of NADPH appear when G6PD activity is below 5% of the normal activity, leading to an immune depression [17]. Vives *et al.* and other groups have also observed an increased susceptibility to infections [18,19].

Preclinical work from Rovira *et al* demonstrates that *hG6PD* gene transfer into HSCs may be a viable strategy for the treatment of severe G6PD deficiency [5]. Through the transplantation of pluripotent hematopoietic stem cells transduced with γ -retroviral vectors carrying the wild type human G6PD cDNA, they achieved a stable and lifelong expression of hG6PD in all the hematopoietic tissues of primary and secondary receptor mice. In this study, transgene expression was driven by the 3' LTR from either the Moloney murine leukemia virus (MMLV) or the myeloproliferative sarcoma virus (MPSV), obtaining an efficient transduction in murine hematopoietic progenitors. The corrected cells were then injected into lethally irradiated syngeneic mice, increasing 2-fold the enzyme activity in peripheral blood cells in comparison with non-transplanted control mice. Long-term hG6PD expression derived from the vector was also observed, which was similar to that of the endogenous enzyme activity. Similar expression was detected in RBC and in White Blood Cells (WBC) in different hematopoietic organs, as expected due to the use of a viral ubiquitous promoter. These results support gene therapy as a suitable strategy for the treatment of severe CNSHA due to G6PD deficiency. Additionally, they also demonstrated the efficacy of this gene therapy vector in human embryonic stem cells (hESC) in which the *G6PD* gene had been inactivated by targeted homologous recombination, which implies the potential application of gene therapy to G6PD hESCs. Moreover, although a selective advantage in favor of G6PD corrected cells has not been reported because the mice used showed normal G6PD activity, Rovira *et al* observed a strong selection after transduction of G6PD-deficient ES cells with their vectors. In this regard, the development of G6PD deficient mouse models would be a valuable tool to test new protocols. Furthermore, the mouse strain recently developed by Hay Ko *et al* may be useful, although it does not reproduce all the features of the human G6PD-deficiency [20].

Pyruvate kinase deficiency (PKD), the second most frequent abnormality of glycolysis causing CNSHA, has also been proposed as a potential disease to be treated by gene therapy. Pyruvate kinase (PK) catalyzes the second ATP generation reaction of the glycolysis pathway by converting phosphoenolpyruvate (PEP) into pyruvate, yielding nearly 50% of the total ATP production in red blood cells. PK plays a crucial role in erythrocyte metabolism, since mature RBC are absolutely dependent on the ATP generated by glycolysis, giving the loss of mitochondria, nucleus and endoplasmic reticulum in their mature state. RPK is therefore necessary for maintaining cell integrity and function. Reduced levels of erythrocyte Pyruvate kinase (RPK) lead to an accumulation of glycolytic intermediates that ultimately shortens the life span of mature RBC by metabolic block [21]. Four tissue-specific isoenzymes of PK (M1, M2, R and L) encoded by two different genes (*PK-M* and *PK-LR*) have been identified in humans [22]. The *PK-LR* gene, located on chromosome 1 (1q21) [23] encodes for both LPK (expressed in liver, renal cortex and small

intestine) and RPK (restricted to erythrocytes) through the use of alternative promoters [24]. PK-M1 is expressed in adult normal tissue, like brain or muscle. The PK-M2 isoform is typically expressed in proliferating tissues like fetal, tumoral and several other adult tissues [25] and during the maturation of the erythroblasts, gradually decreases, giving rise to the RPK isoform.

The coding region of *PK-LR* gene is split into twelve exons, ten of which are shared by the two isoforms, while exons 1 and 2 are specific for the erythrocyte and the hepatic isoenzyme respectively [26]. However, clinical symptoms caused by *PK-LR* mutations are confined to RBC because the hepatic deficiency is usually compensated by the persistent enzyme synthesis in hepatocytes [27]. To date, more than 150 different mutations in the *PK-LR* have been associated with CNSHA, being most of them missense mutations, splicing and codon stop. Only two variants, -72 G and -83 C, have been identified in the promoter regions so far [26,27]. Molecular studies indicate that severe syndrome is commonly associated with disruptive mutations and missense mutations involving the active site or protein stability [28].

PK deficiency is transmitted as an autosomal recessive trait and although its global incidence is still unknown, it has been estimated in 1:20000 in the general caucasian population [29]. Clinical symptoms appear in homozygous and compound heterozygous patients, which lead to a very variable clinical picture, ranging from mild or fully compensated forms to life-threatening neonatal anemia necessitating exchange transfusions and subsequent continuous support [28]. Pathological manifestations are usually observed when enzyme activity falls below 25% of normal PK activity [30], and severe disease has been associated with a high degree of reticulocytosis [31]. *Hydrops foetalis* and death in the neonatal period have also been reported in rare cases [32,33]. PK deficiency treatment is based on supportive measures since no specific therapy for severe cases is available to date. Periodic cell transfusions may be required in severe anemic cases, often impairing their quality of life. Splenectomy can be clinically useful in some patients increasing the hemoglobin levels, as well as iron chelation to decrease the common iron overload observed in PKD patients [34]. However, in some severe cases, allogeneic bone marrow transplantation is required and it has been successfully performed in one severe affected child [12].

The feasibility of gene therapy in PKD was first reported by the group of Asano, who introduced the human LPK cDNA into C57BL/6 mouse bone marrow cells using a retroviral vector [7]. They demonstrated the expression of the LPK transgene mRNA in both peripheral blood and hematopoietic organs after bone marrow transplantation. However, viral-derived expression in peripheral blood was detectable no longer than 30 days post-transplantation, indicating an insufficient transduction efficacy of the retroviral vector used or transduction of non-pluripotent BM cells. In a hemolytic anemia dog model, bone marrow transplantation of minimal conditioned receptors failed to correct the hematological symptoms [10]. Other approaches to rescue RPK phenotype through a gene addition strategy have been also addressed using a PKD transgenic mouse model (*CBA/N PK-1^{SLC}/PK-1^{SLC}*) [9]. In this assay, the hemolytic anemia and reticulocytosis was fully corrected when the human gene was highly expressed by means of pronuclear injection, although splenomegaly was still present. Interestingly, the authors observed a negative correlation between RBC PK activity and the number of apoptotic erythroid progenitors in the spleen, providing evidence that the meta-

bolic alteration in PK deficiency affects not only the survival of RBC, but also the maturation of erythroid progenitors, resulting in ineffective erythropoiesis [35]. Further studies from this group indicate that RPK plays an important role as an antioxidant during erythrocyte differentiation, since glycolytic inhibition by mutations in *Pklr* gene increased the oxidative stress in SLC3 cells (established from *Pk-1^{slc}* mouse) and led to the activation of hypoxia-inducible factor-1 (HIF1), as well as the expression of downstream proapoptotic genes [36].

In addition, our work carried out in mouse models supported the therapeutic potential of viral vectors for the gene therapy of PK deficiency. Throughout the transduction of bone marrow cells using γ -retroviral vectors that carry the human RPK cDNA and subsequent transplantation, we reported a long-term expression of the human protein in RBC obtained from primary and secondary receptor mice, without detectable adverse effects [11]. Recently, we have also reported a successful gene therapy approach using the same retroviral vectors in the congenital mouse strain AcB55, identified by Min-Oo in studies of alleles involved in malaria susceptibility [37]. These mice carry a loss-of-function mutation (269T-> A) resulting in the amino acid substitution I90N in the *Pklr* gene, which yields a similar RBC phenotype to that observed in PKD patients, including splenomegaly and constitutive reticulocytosis. Retroviral-derived expression was capable of fully resolving the pathological phenotype in terms of hematological parameters, anemia, reticulocytosis and splenomegaly, together with normalization of bone marrow and spleen erythroid progenitors, erythropoietin (EPO), PK activity and ATP levels. Interestingly, despite a strong viral promoter was used to drive the expression of the transgene, metabolic energy balance was not modified in white blood cells. Moreover, we observed that values above 25% of genetically corrected cells were needed to fully rescue the deficiency [3], suggesting that RPK transfer protocols will always require a significant extent of gene-complemented HSC. Nevertheless, other experiments performed in the *CBA/N PK-1^{SLC}/PK-1^{SLC}* mouse model of PKD have revealed that 10% of normal BM renders RBC expressing nearly normal RPK protein levels [5]. Differences in the genetic defect of the mouse models used could account for these discrepancies, reinforcing the need for high transduction efficiencies to address the disease in the heterogeneous human population. Additionally, we have proposed the *in utero* transplantation of gene corrected cells as an alternative option for the treatment of PKD. The transplantation of RPK deficient lineage negative fetal liver cells transduced with lentiviruses (LVs) expressing the human wild type version of the RPK in 14.5 day-old fetuses partially restored the anemic phenotype, mainly due to a low engraftment of corrected cells [13]. Improved *in utero* cell transfer would allow therapeutic levels, thus offering an alternative therapeutic option for prenatally diagnosed severe PKD. Following our results in the AcB55 mouse model of PKD, phenotype correction could be reached if the percentage of engraftment of corrected cells is significant. We are currently developing improved lentiviral vectors that could be applied in future clinical settings.

Glucose phosphate isomerase (GPI) deficiency is the third most common hereditary cause of CNSHA, due to mutations in *GPI* gene located on the long arm of chromosome 19. The prevalence of this disease is still unknown, with no more than 50 cases reported so far, and with a higher incidence in the black population. The enzyme catalyzes the reversible isomer-

ization from glucose 6-phosphate to fructose 6-phosphate, an equilibrium reaction of the glycolysis pathway. Glucose turnover is affected only in deficiencies below a very low critical residual GPI activity, but with a drastic decline of lactate formation. As no isoenzyme does exist, patients suffer not only from CNSHA and tissue hypoxia, but also from neuromuscular disturbances. In some cases, GPI deficiency has been found in PKD patients, increasing the severity of the clinical scenario and reflecting the degree of the perturbation of glycolysis. The lack of ATP leads to a destabilization of the erythrocyte membrane causing earlier lysis of the RBC and hemolytic anemia of variable degrees [38]. Animal models of GPI deficiency have been described, showing similar symptoms to the human disease [39]. Until now, no gene therapy attempt has been applied to this deficiency.

Other enzyme deficiencies causing CNSHA are Triose phosphate isomerase (TPI) deficiency, associated with neuromuscular disorders, mental retardation and frequent infections, Hexokinase deficiency (HK), affecting also platelet metabolism, phosphofructokinase (PFK) deficiency, 2,3-bisphosphoglycerate mutase (BPGM) deficiency and Glutathione synthetase (GS) deficiency (reviewed in [17,40,41]). Although the incidence of these diseases can be high (ie. TPI is considered as a frequent enzymopathy affecting 0.1% for caucasian populations and even 4.6% for black populations), they are considered rare or very rare diseases, because only few cases (~25 patients in the case of TPI) are diagnosed due to the severity of the clinical manifestations. No gene therapy approaches have been addressed up to now to treat these enzymopathies. However, due to their common characteristics, strategies developed in the other enzyme deficiencies could be applied directly to the treatment of all of them.

3. Optimization of vectors for the gene therapy of metabolic erythroid diseases: Erythroid specific expression vectors

The introduction of a cDNA, encoding for the correct version of the target mutated gene into patient cells using retroviral vectors has been successful for several inherited diseases. The initial integrative vectors for gene therapy design and used in clinical trials were based on Gamma(γ)-retroviral vectors in which the transgene expression was driven by the viral LTR promoter. γ -retroviruses preferably integrate in regions adjacent to the transcription initiation site [42]. The expression of the transgene is promoted by the viral LTR, which drives a high expression that can affect gene regulation of the surrounding genes. Although a high efficiency of transduction and therapeutic effects have been described with these vectors in various monogenic disorders such as immunodeficiencies, adverse effects associated with insertional mutagenesis have also been observed. This has led to the development of the next generation of integrative vectors using self-inactivating-LTR lentiviral backbones. SIN-Lentiviral vectors tend to integrate in intergenic transcribing areas, which represent a safer integrative pattern than γ -retroviral vectors. Additionally, the expression of the transgene is driven by internal promoters, offering a more physiological expression and a less genotoxic profile when using weak promoters [43]. Current efforts to reduce the mutagenic potential of gene therapy vectors are focussed on not only the use of new viral backbones [44] but also on tissue-specific promoters to restrict the transgene expression to target cells [45] and insu-

lators to confer position-independent expression [46]. Additional regulatory DNA elements such as locus control regions (LCR), enhancers, or silencers have also been used to increase lineage specificity.

Gene therapy for RBC disorders requires, ideally, high erythroid-specific transgene expression in order to avoid side effects in progenitors or hematopoietic lineages other than the erythroid one. In inherited enzymopathies, the overexpression of metabolic enzymes in non-erythroid cells could provide these cells with a potential energetic advantage, with the consequent risk of disturbing the physiological generation of ATP in WBC. Also, the restriction of transcriptional activity to target cells with the use of either tissue-specific or physiologically regulated vectors decreases the effect of the integrative vectors in the host genome. This goal is particularly important for erythrocyte metabolic deficiencies, as all the affected enzymes are highly regulated and connected with central metabolic pathways. Indeed, an expression limited to the erythroid progeny would reduce the genotoxic risk, as RBC become transcriptionally inactive during differentiation, and finally extrude their nucleus. To study tissue-specific gene therapy strategies for RBC diseases, hemoglobinopathies have been the most widely used.

Erythroid regulatory elements have been extensively used to manage targeted expression to RBC using reporter genes (Table 2). The Locus Control Regions (LCR), defined by their ability to enhance the expression of linked genes to physiological levels in a tissue-specific and copy number-dependent manner at ectopic chromatin sites are commonly used. The components of the LCR normally colocalize to sites of DNase I hypersensitivity (HS) in the chromatin of expressing cells. Individual HS are composed of arrays of multiple ubiquitous and lineage-specific transcription factor-binding sites. In early experiments performed with retroviral backbones, the group of Ferrari developed an erythroid-specific vector by the replacement of the constitutive retroviral enhancer in the U3 region of the 3' LTR with the HS2 autoregulatory enhancer of the erythroid GATA-1 transcription factor gene. The expression of this vector was restricted to the erythroblastic progeny of both human progenitors and mouse-repopulating stem cells [47,48]. Later, they showed that the addition of the HS1 enhancer to HS2, both from the GATA-1 gene, within the LTR of the retroviral vector significantly improved the expression of the reporter gene. Another enhancer element that has been used to achieve erythroid-specific expression is HS40, located upstream of the ζ -globin gene, since it is able to enhance the activity of heterologous promoters in a tissue-specific manner [49]. It has been shown to be genetically stable in MMLV vectors and enhances expression comparable to that of a single -globin gene [50], although HS40 lacks some of the properties of the LCR, like position independence [51] or copy number dependence [52].

An additional improvement to provide safer vectors for RBC gene therapy was provided by the use of insulators elements, which have been shown to reduce position effects in transgenic animals [60]. Insulators are genomic elements that can shelter genes from their surrounding chromosomal environment, by either blocking the action of a distal enhancer on a promoter [60,61], or by acting as barriers that protect the gene from the silencing effect of heterochromatin [61]. The most well studied element is the chicken hypersensitive site 4

(cHS4), an insulator sequence of the chicken γ -like globin cluster. Studies performed by Chung et al with the γ -globin promoter and the neo reporter gene on selected cells lines, demonstrated the ability of cHS4 to insulate the expression cassette from the effects of a strong γ -globin LCR element [63] and therefore reducing its genotoxicity. Experiments from Arumugam et al showed a two-fold reduction in transforming activity with insulated LCR-containing lentiviral vectors comparing with vectors lacking the cHS4 element [68].

Erythroid tissue-specific vectors			
Promoter / enhancer	transgene	Vector type	Reference
HS2 GATA-1 enhancer within the LTR	Δ LNGFR and Neo ^R / EGFP	SFCM retroviral vector	[47]
HS1 to HS2 GATA-1 enhancer within the LTR	EGFP and h Δ LNGFR	SFCM retroviral vector	[48]
Ankyrin-1 and α -spectrin promoters combined or not with HS40, GATA-1, ARE and intron 8 enhancers	EGFP	HIV-1 based vectors	[53]
α -globin HS40 enhancer and Ankyrin-1 promoter	GFP / FECHcDNA	HIV-1 based vectors	[4]
IHK, IH β p and HS3 β p chimeric enhancers/ promoters	h β -globin cDNA	Sleeping beauty transposon	[54]
Physiologically regulated vectors			
Promoter / enhancer	transgene	Vector type	Reference
HSFE and β -globin promoter	h β -globin cDNA	MSCV retroviral vector	[55]
LCR and β -globin promoter	h β -globin cDNA or EGFP	HIV-1 based vectors	[56,57]
β -globin and θ -globin promoters combined or not with HS40, GATA-1, ARE and intron 8 enhancers	EGFP	HIV-1 based vectors	[53]
LCR HS4, HS3, HS2, β -globin promoter and truncated β -globin intron 2	EGFP	HIV-1 based vectors	[58]
LCR, cHS4 and β -globin promoter	h β -globin cDNA	HIV-1 based vectors	[46]
β -globin promoter, LCR HS2, HS3, HS4	h β -globin cDNA	AAV2	[59]

LTR, long terminal repeats; HS: hypersensitive site; IHK, human *ALAS2* intron 8 enhancer, HS40 from α LCR and ankyrin-1 promoter; IH β p, human *ALAS2* intron 8 enhancer, HS40 from α LCR and β -globin promoter; HS3 β p, HS3 core element from human β LCR and β -globin promoter; LCR, locus control region. Modified from Toscano et al., 2011

Table 2. Specific vectors for gene therapy of erythroid inherited diseases.

Tissue-specific expression using alternative human promoters can be convenient or more efficient for some diseases, but driving the expression of the therapeutic genes using own promoters is still the most physiological approach to reduce the genotoxic risk of integrating gene vectors [62]. The use of physiologically regulated vectors has been limited mainly because the promoter and the enhancer elements have to be obtained from the affected genes and they are often too large to be included in a lentiviral backbone, and also because the gene expression pattern depends partially on chromatin positioning [63]. γ -globin LCR has been widely used when attempting to solve this limitation. The γ -globin LCR consists of 5 HS regions located upstream of the entire cluster of human γ -like globin genes, each containing a high density of erythroid-specific and ubiquitous transcription binding elements [64]. Much of the transcriptional activity of the γ -globin LCR resides in HS2 and HS3 sites, but site 4 is important in adult globin expression [65]. Previous studies *in vitro* and *in vivo* have shown that γ -globin LCR can enhance erythroid-specific expression from heterologous non-erythroid promoters [66,67]. First approaches using γ -globin LCR and 3' enhancers were based on murine γ retroviral vectors [74,75], but the limited packaging capacity of these vectors (up to 8 kb) did not allow the presence of such as large regulatory sequences. Several vector designs including different combinations of regulatory sequences and a deletion of a cryptic polyadenylation site within intron 2 of γ -globin gene [68], flanked by an extended promoter sequence and the γ -globin 3' proximal enhancer were developed. The combination of the LCR elements (3'2 kb) spanning HS2, 3 and 4, were the best amongst several possibilities [69] to achieve a high titer retroviral vector capable of expressing high levels of the transgene.

Other approaches to achieve consistent long-term expression of a transgene have been based on the use of HSFE element, an erythroid-specific chromatin remodelling element derived from the human β -globin LCR which contains binding sites for the erythroid-specific factors NF-E2, GATA-1, EKLF and the ubiquitous factor Sp-1, all of which are necessary to establish a hypersensitive chromatin domain. Work by Nemeth *et al.*, demonstrated that the HSFE can mediate functional tissue-specific "opening" of a minimal human β -globin promoter and increases expression of a human β -globin gene in both MEL cell clones and in transgenic mice. Their results indicated that the most effective vector included tandem copies of the HSFE and produced a 5-fold increase in expression compared to the promoter alone [55] in the context of an integrated retroviral vector.

Gene therapy for RBC metabolic diseases can also benefit from the new technologies based on the modification in mRNA stability or translation efficacy of the transgenes. The use of the post-transcriptional regulatory element (Wpre) from the woodchuck hepatitis virus (WHV) has significantly increased transgene expression in target cells [64,65], even in HSC [70] by stabilization of mRNA at post-transcriptional level. However, it may raise safety concerns, since it contains a truncated form of the WHV X gene, which has been implicated in animal liver cancer [71]. Therefore, Wpre has subsequently been improved by a mutation of the open reading frame of the X gene [72]. Combination of erythroid promoters like ankyrin-1 or -spectrin with Wpre sequence increased 2-fold the expression in unilineage erythroid cultures [53], and when combined also with erythroid enhancers inserted in tandem: HS40 and GATA-1 or HS40 and I8 enhancers [53]. RNA targeting strategies have mainly been used to down regulate expression of cellular genes using vectors expressing interference RNAs (iRNAs). They can be also used to control the expression of integrating vectors knocking down the transgene by the

endogenous microRNA cellular machinery. Following this strategy, engineered microRNA target sequences in the vector (miRTs-vector) are recognized by a cell specific microRNA (miRNA), avoiding the expression of the therapeutic gene in undesired cell populations [63]. Several miRNAs are differentially expressed during hematopoiesis and their specific expression regulates key functional proteins involved in hematopoietic lineage differentiation. Particularly, miR-223 has been proposed as a myeloid-specific regulator that negatively regulates progenitor proliferation and granulocyte differentiation and activation [73]. Moreover, Felli et al observed that hematopoietic progenitor cells transduced with miR-223 showed a significant reduction of their erythroid clonogenic capacity, suggesting that down-modulation of this miRNA is required for erythroid progenitor recruitment and commitment [79]. Further studies may determine if the use of miRNA-223 target in lentiviral vectors could be useful to achieve a desirable erythroid-specific expression for gene therapy of red blood cell diseases.

In addition, the erythroid-specificity of short segments of the γ -globin LCR element has been documented in adeno-associated virus 2 (AAV2) system. Their efficacy to mediate an erythroid-restricted expression has been proved by Tan *et al.*, who reported a successful AAV2-mediated high and stable transduction of the human γ -globin gene in HSCs from α -thalassemia mouse model, which were then transplanted into recipient and rescued them of the disease [59]. These vectors have gained attention as potential useful vectors for human gene therapy, mainly because of their non-pathogenic nature in humans and their relatively easy production. Besides, AAV2 vectors are easily purified to high titers and are able to transduce dividing and non-dividing cells. However, most of proviral AAV2 genomes remain episomal and the insert size is restricted to just over 4kb. Further studies are still needed to know whether they would be a better option than current lentiviral vectors. Also, long-term genotoxic risk of recombinant AAV2 therapy in human is not known up to the date.

In addition, the efficacy of some of these erythroid-specific elements and promoters has also been tested in non-viral vectors, such as transposons. Zhu et al, for instance, studied several hybrid promoters driving the expression of the human γ -globin gene using the sleeping beauty transposon (SB-Tn). They combined several erythroid elements to develop different chimeric promoters. Their results indicated that the ankyrin-1 minimum promoter was stronger than γ -globin's, and the hALAS I8 enhancer (IH) was significantly more powerful than HS3 core element from γ -LCR and γ -globin promoter [54]. SB-Tn system is a promising non-viral vector for efficient genomic insertion, even with erythroid-specificity. However, its efficiency for delivering transgenes into HSCs is still much lower than other engineering viral vectors.

4. Overcoming conventional gene therapy pitfalls: gene editing in induced pluripotent stem cells

4.1. Human induced pluripotent stem cells and reprogramming platforms

Since Yamanaka et al first reported the generation of mouse induced Pluripotent Stem Cells (iPSC) in 2006 by the ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and

cMyc) [74] and one year later in human cells together with Thompson's group [75,76], many laboratories around the world have been able to reprogram a large range of somatic cells into pluripotent stem cells, from neural stem cells [77] to terminally differentiated B-lymphocytes [78]. The reproducibility and potentiality (unlimited self-renewal and ability to differentiate into any cell type) of this technology has made the iPSC field to advance very rapidly. The human iPSC (hiPSC) technology brings together all the potential of hESC in terms of pluripotency without any ethical issue and the immunotolerance of the autologous cell treatment. Therefore, hiPSC technology is one of the most promising fields for future therapies for many human diseases. Safer reprogramming approaches have been designed and many patient specific hiPSC have been generated both to model human diseases and to correct by gene therapy approaches. Depending on the cell type to be reprogrammed, the number of factors used could be reduced and, what is more important, oncogenes or tumor related proteins included in the reprogramming cocktail, like *c-MYC* or *KLF4* [79] could be removed from the original reprogramming cocktail [80-82]. Several groups developed excisable polycistronic lentiviral vectors [83,84] or transposon-based reprogramming systems [85,86], which could be removed after getting the hiPSC clones. Similar results have been obtained using recombinant proteins [82], synthetic mRNAs [87], and non integrating RNA Sendai Virus vectors [88]. Except for Sendai viruses, non integrating methods show a reduced reprogramming efficiency and the range of cells reprogrammed is not as large as with lentiviral or retroviral vectors.

iPSC technology makes feasible the availability of patient specific cells to study the biology of the disease and develop advanced tools to cure the phenotype and could potentially be used as a therapeutic option (Figure 1). Focussing on metabolic diseases, the first reported metabolic disease patient specific hiPSC line was obtained one year after the first generation of hiPSCs. It was from a 42-year old female that suffered from Type I Diabetes mellitus [89] and it showed no differences compared to a wild type hiPSC line in terms of pluripotency. Next report in which liver metabolic disease patient samples were reprogrammed was carried out by the group of Ludovic Vallier [90], and showed the potential of this kind of approaches for disease modelling and new drug discovery. They reprogrammed fibroblast obtained from α -1 Antitrypsin deficiency (*A1ATD*), Familiar Hypercholesterolemia (FH), Glucose-6-Phosphate deficiency (G6PD), Crigler-Najjar Syndrome and hereditary Tyrosinemia Type 1 patients, and generated hepatocytes that showed characteristics of mature hepatic cells, like albumin secretion or cytochrome p450 metabolism. Three of the five cell lines (*A1ATD*, FH, and *GSD1a* hiPSCs) were capable of recapitulating the disease phenotype in vitro. Disease modelling in erythroid diseased induced pluripotent cell lines has been performed for β -Thalassemia [91,92] and sickle cell anemia [93,94]. In these reports the phenotype was corrected by LVs integrated in areas of the genome that were considered safe for viral integration [83] or by gene editing using homologous recombination of the affected locus [91,93,94].

The future therapeutic application of hiPSC will not only require non-integrative reprogramming system, but also a more precise gene correction. During last years, the cooperation between hiPSC technology and gene editing is being explored. Human iPSC technology has

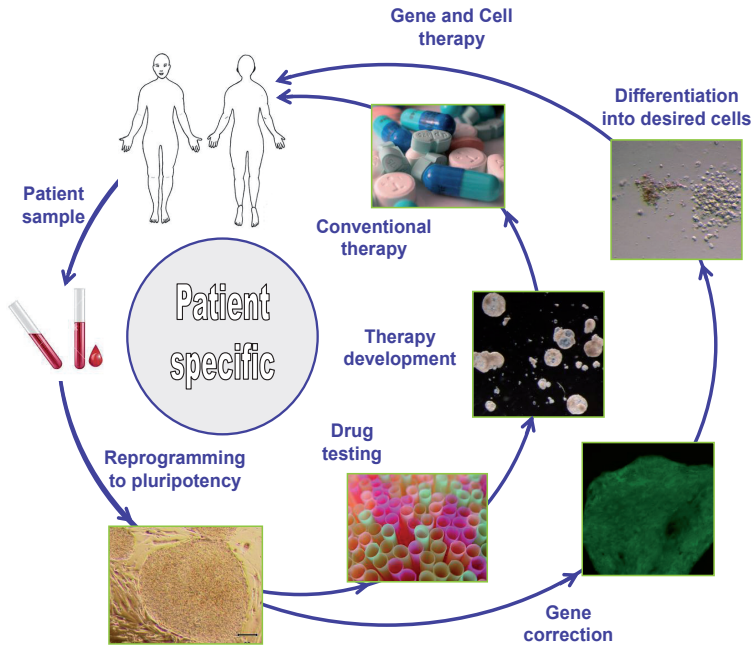


Figure 1. Potential utilities of hiPSC and iPS technology

led to the opportunity to control the integration of viral vectors at a clonal level. As we have mentioned before, the analysis of lentiviral integration sites in β -thalassemia hiPSC allowed the identification of corrected hiPSC clones expressing β -globin transgene from a safe genomic site (also called Safe harbour), a site in which integration does not disturb the expression of any neighbouring genes during their erythroid differentiation [83]. The therapeutic use of patient-specific hiPSC emerges then from the combination of gene and cell therapy. From this new research field, future gene therapy protocols will emerge.

4.2. Gene editing based on homologous recombination

Gene editing is a process in which a DNA sequence is introduced into a specific locus or a chromosomal sequence is replaced. This site-specific precise introduction requires an accurate recognition mechanism of the target site on the genome. Under normal conditions, the maintenance of the integrity of the genome requires that the cells repair DNA damage with high fidelity. One of the most harmful DNA damage is the generation of double-strand breaks (DSB). DSB are often resolved by non-homologous end joining (NHEJ), which joins the two ends of the DSB. However this DNA repair mechanism could introduce mutations. On the contrary, homologous recombination (HR) is a truly accurate DNA repair mechanism because it is basically a “copy and paste” mechanism. This process uses an undamaged

homologous segment of DNA that can be exogenously provided as a template to copy the information across the DSB. The fidelity of HR gives us the specificity and accuracy that gene editing requires.

The natural HR process has been adapted by researchers to get the desirable addition of an exogenous cassette into the targeted locus. This technique has been widely used for the generation of knock-out and knock-in transgenic animals [95]. To correct or insert and express a transgene by HR we can consider three different strategies: i) Gene correction, a base or some bases can be substituted from the original strand using an homologous sequence where this base or bases are modified; it is the way to introduce/repair point or small mutations; ii) Safe harbour integration, a complete expression cassette (promoter, transgene and regulatory signals) is inserted in a safe place of the genome, without altering the expression of the surrounding genes and without being silenced by epigenetic mechanisms; this is the case for *AASV1* and *CCR5* loci. Additionally to these well known safe harbours, there is a wide research focused on finding potential new safe harbour places. iii) Knock-in insertion, the cDNA of a gene is introduced in the same site of the endogenous gene, linked by splicing mechanisms to the endogenous gene assuring the expression of the inserted sequence by the endogenous regulatory elements of the locus where it is integrated.

Gene editing process can be separated in two different steps, generation of DSB and HR. The efficacy of gene editing in human cells depends on the generation of DSB at the specific target site and on the DNA repair mechanism that the cell uses to resolve the DSB. Unfortunately, NHEJ is the dominant pathway to solve these DNA lesions in human cells. Additionally, HR varies in different cell types and requires transit through S-G2 phase of the cell cycle [96]. These limitations make gene editing in human cells difficult to achieve. However, different approaches are being used to improve gene editing by HR, like increasing the length of the DNA sequences homologous to target site (homology arms) [97], the use of adeno-associated vectors [98], the improvement of selection methods for edited cells or the stimulation of HR by inducing DSB using DNA nucleases.

Recently, engineered DNA nucleases have been developed to specifically induce DSB at a unique and defined sequence in the cell genome. These proteins are formed by a nuclease domain and a DNA binding domain whose sequence specificity can be engineered. The most widely used DNA nucleases are Zinc finger nucleases (ZFN), homing meganucleases (MN) and transcription activator-like effector nucleases (TALEN). They identify a potentially unique sequence in the genome and generate DSBs in the desired genomic site, aiming to promote the repair of the DSB by the cell machinery and, ideally by HR. The DNA binding domain of a ZFNs is derived from zinc-finger proteins and is linked to the nuclease domain of the restriction enzyme Fok-I. DNA-binding domain is a tandem repeat of Cys₂His₂ zinc fingers, each of which recognizes three nucleotides. ZFNs work as pairs of two monomers of ZFN, one in reverse orientation. This ZFN dimer can be designed to bind to genomic sequences of 18-36 nucleotides long. TALENs have a similar structure to ZFNs, but the DNA-binding domain comes from transcription activator-like effector proteins. The DNA-binding domain in TALENs is a tandem array of amino acid repeats. Each of these units is able to bind to one of the four possible nucleotides and this makes that the DNA binding domain can be designed to recognize any desired genomic sequence. TALENs also cleave as dimers. Contrary to these synthetic DNA-nucleases, MNs are a

subset of homing endonucleases which recognize a DNA sequence from 14 to 40 nucleotides. Current MNs have been engineered from natural homing endonucleases to increase the number of target DNA sequences.

ZNFs have been widely used for gene editing in hESC and hiPSC. In 2007, Dr. Naldini's laboratory showed the insertion of GFP into the CCR5 safe harbour in human stem cells (hESC and hiPSC) after inducing HR by ZFN expression. The CCR5-ZFN and donor DNAs were delivered into hESC by integrative deficient lentiviruses. More interestingly, targeted hESC were able to differentiate into neurons keeping GFP expression [99]. Soon, the proof of principle for the clinical application of ZFN-mediated gene editing was tested in hiPSC from patients affected by different genetic diseases. The first pre-clinical use of ZFN for gene therapy of a metabolic disease was performed by Yusa *et al.* In this report, gene correction was performed at the α 1-antitrypsin (A1AT) locus to revert A1AT deficiency in hiPSC derived from a patient with a point mutation. This group included a Puromycin resistance cassette flanked by piggyBac sites, so that the Puromycin selection facilitated the isolation of corrected A1ATD-iPSC clones. Afterwards, the selection cassette was removed by piggyBac transposon, obtaining corrected hiPS clones without any additional sequence. These corrected hiPS clones were then differentiated into hepatocyte-like cells to confirm the complete correction of the A1ATD [101]. Other hiPSC gene editing approaches and functional correction of erythroid diseases include gene correction of Sickle Cell Anemia [94] and β -Thalassemia [91].

One of the major limitations of ZFN is the generation of "off-target" DSB, due to unspecific sequence recognition. Different studies have highlighted this as a possible limitation in the clinical use of ZFN-mediated HR [100,101]. Recent works have explored the potential of other types of DNA-nucleases in order to prevent the "off-target" cleave limitations of the ZFN, being TALEN and MN the most promising ones. The feasibility of TALEN to mediate HR in hESC and hiPSC was assessed by Jaenisch's group when they designed TALEN targeting the *PPP1R12C* (at *AAVS1* locus), *POU5F1* and *PITX3* genes at precisely the same positions as the one targeted by ZFN in their previous work [102]. The authors described a gene editing efficiency similar to the one achieved by ZFN with a low level of "off-targets" [103]. A strategy to minimize the potential number of "off-targets" is to design TALEN to work as obligatory heterodimers, which has been already done in the engineered MNs. The application of the TALEN and MN as tools to improve HR is still on going. We are exploring the pre-clinical use of TALEN and MN to correct erythroid metabolic genetic diseases, such as PKD.

5. Complementary developments for the application of gene therapy to erythroid metabolic diseases

5.1. *In vivo* transduction using engineered envelopes

Another challenge for the clinical application of gene therapy relates to vector targeting. To achieve successful gene therapy, the appropriate gene must be delivered to target cells and specifically expressed in them, without harming non-targeted cells. The most common and easiest way to target specific cells is by *ex vivo* infection of the desired cell population. There-

fore, cells can be directly exposed to the viral vectors facilitating viral-cell interaction. These interactions are driven by the envelope protein which can be adapted from other viruses re-directing the tropism of the vector. The most widely used vectors are lentiviral vectors pseudotyped with the attachment glycoprotein of the vesicular stomatitis virus (VSV-G), which allows the production of high-titre vectors and confers a broad host range [104]. In comparison with them, engineered LVs capable of delivering genes of interest to predetermined cells, can reduce the targeting of undesirable cell types and improve the safety profile, which will further enhance the use of this vector system for gene therapy applications [105,106]. As we have mentioned above, the use of promoters and regulatory sequences that are only active in target cells adds lineage specific expression, although integration of the viruses in non desired cells is still possible. *Ex vivo*-targeted gene delivery, as commonly used in HSCs transduction, is associated with a risk of inducing cell differentiation and loss of the engraftment potential of these cells [107]. On the contrary, *in vivo* gene transfer could target HSCs in their stem cell niche, a microenvironment that regulates HSC survival and maintenance [105]. To accomplish this, the vector must display a suitable system to selectively infect the desired population, for example the introduction of a specific ligand to bind a target-cell receptor [106].

Many attempts have been made to develop targeted transduction systems using retroviral and lentiviral vectors by altering the envelope glycoprotein (Env), which is responsible for the binding of the virus to the cell surface receptors and for mediating viral entry into the cell. The plasticity of the surface domain of Env allows insertion of ligands, peptides or single-chain antibodies that can direct the vectors to specific cell types [108]. However, this type of manipulation negatively affects the fusion domain of Env, resulting in low viral titers. To overcome this downside, a method to engineer lentiviral vectors has been developed. These vectors transduce specific cell types by breaking up the binding and fusion functions of the envelope protein into two distinct proteins [108]. Instead of pseudotyping lentiviral vectors with a modified viral envelope protein, these lentiviral vectors co-display a targeting antibody and a fusogenic molecule on the same viral vector surface. Based on molecular recognition, the targeting antibody should direct lentiviral vectors to the specific cell type. The binding between the antibody and the corresponding cellular antigen should induce endocytosis resulting in the transport of lentiviral vectors into the endosomal compartment. Once inside the endosome, the fusogenic molecule should undergo a conformational change in response to the decrease in pH, thereby releasing the viral core into the cytosol [109]. The use of fusion domain of the binding defective Sindbis virus glycoprotein together with an anti-CD20 antibody has been shown to mediate the targeted transduction of lentiviral vectors to CD20-expressing B cells [110].

However, two major challenges for *in vivo* gene delivery are LVthe exposure to the host immune/complement system and off-target cell transfer after systemic administration. For these reasons, second generation of early-acting-cytokine-displaying LVs has been developed, that circumvents these obstacles by specifically targeting hCD34⁺ cells [111,112]. For example, RDTR/SCFHA-LV, consisting of RD114 glycoprotein and stem cell factor (SCF) fused to the *Influenza hamagglutinin* env protein, is resistant to degradation by human comple-

ment and efficiently transduces very immature hCD34⁺ HSCs [113]. This new generation of HSC-targeted LVs should improve current gene therapy protocols through the transduction of primitive HSCs directly in the bone marrow of patients with genetic diseases.

5.2. *In vitro* production of mature erythrocytes

Periodical blood transfusion is the previous to the last therapeutic option for severe cases of CNSHA patients. However, this clinical practice involves also adverse effects related to the immuneresponse against minor erythrocyte antigens which makes the patients refractory to additional blood transfusions in the long run. The availability of genetically corrected patient-specific iPSC would allow the possibility of generating disease free erythrocytes ready for transfusion, avoiding the adverse immune effects.

There have been numerous attempts to produce RBC *in vitro* from different sources of stem cells. To date, the most successful protocol has been developed by the group of Luc Douay [113,114]. Using peripheral blood CD34⁺ cells, these authors were able to expand and generate RBC with *in vitro* and *in vivo* features of native RBC, and were also capable of transfusing a patient with *in vitro* generated erythrocytes. Notably, the same authors reported a protocol to generate RBC from hiPSC as an alternative source of HSC [114]. Other groups have described similar protocols to generate erythrocytes from hESC or hiPSC [115-118], although in all these studies the RBC generated from embryonic cells expressed embryonic and foetal hemoglobins but low levels of adult hemoglobin. Additional efforts should be done to make this possibility a therapeutic option.

6. Conclusions

Erythroid metabolic diseases are well defined and well known diseases which main symptom is CNSHA. As they are monogenic diseases that can be cured by allogeneic bone marrow transplantation, they are very good candidates to be treated by gene therapy. However, the low number of patients with poor prognosis requiring BM transplantation and the absence of an apparent selective advantage of the corrected cells over the diseased ones have made their approach for gene therapy less attractive than other erythropaties. Up to now, no gene therapy clinical trial for erythroid metabolic diseases has been accomplished. Gene therapy attempts in animal models have been applied to G6PD and PKD with successful results, emphasizing the usefulness of a gene therapy approach for these diseases. Although adverse effects due to ectopic expression of the metabolic enzyme have not been observed, an erythroid specific expression is preferred. Many developments have been made for the specific expression of globin genes that could be adapted to vectors developed for the discussed erythroid metabolic diseases. Similarly, any attempt directed to the improvement of HSC transduction, including the possibility of *in vivo* targeted gene therapy could be applied. On the other hand, the combination of cell reprogramming and gene editing opens a new world of possibilities that could be easily applied to these diseases. hESC and hiPSC are helping in the development of the next generation of gene therapy, which implies a precise

gene targeting. Gene editing by HR is the best and safest gene therapy procedure because avoids any perturbation in the targeted genome. Besides the combination of hiPSC and gene editing could be the future therapy for many genetic-based diseases. The hiPSC technology is the springboard for the development of more efficient HR protocols applicable to other types of stem cells such as hematopoietic stem cells. The combination of methods for obtaining big amounts of RBC from HSC or embryonic cells, along with the improvement of the different gene therapy approaches described in this chapter, opens up the possibility of the therapeutic application involving the infusion of RBC differentiated *in vitro* from genetically corrected patient specific stem cells.

Nomenclature

5-FU 5-fluorouracil

A1ATD-1 antitripsin deficiency

AAV Adeno-associated virus

BM Bone marrow

BPGM 2,3-bisphosphoglycerate mutase

CNSHA Chronic non spherocytic hemaolytic anemia

DSB Double strand breaks

Env Viral envelope

FH familial hypercholesterolemia

G6P Glucose-6-phosphate

G6PD Glucose-6-phosphate dehydrogenase

GPI Glucose phosphate isomerase

GS Glutathione synthetase

hESC human embryonic stem cell

hIF1 hypoxia-inducible factor-1

hiPSC Human induced pluripotent stem cell

HK Hexokinase

HR Homologous recombination

HS DNase I hypersensitive sites

HSC Hematopoietic stem cell

iPSC Induced pluripotent stem cell

kb kilobases
LCR Locus control region
LTR Long terminal repeats
LV Lentivirus
MN homing meganuclease
NHEJ non-homologous end joining
PFK phosphofructokinase
RBC Erythrocytes
SIN-LV Self-inactivated lentiviral vector
TALEN transcription activator-like effector nuclease
TPI Triose phosphate isomerase
WT wild-type
ZFN zinc finger nuclease

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Targeting the Lung: Challenges in Gene Therapy for Cystic Fibrosis

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Additional information is available at the end of the chapter

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1. Introduction

Cystic Fibrosis (CF) is the most common fatal autosomal recessive genetic disease in the Caucasians with a frequency of approximately 1 in 2500 newborns (Cystic Fibrosis Foundation, <http://www.cff.org/>). It affects several organs including the lungs, the liver, the pancreas, the sweat glands and the gastrointestinal and reproductive tracts [1]. The most severe complications that finally lead to death are those in the airway epithelium [2]. Continuous secretion of mucus causes blockage of the lungs by thick sputum and also makes the lungs susceptible to secondary bacterial infections. Subsequent inflammatory responses by the immune system damage the lungs and the combination of all these factors leads to cardiac failure and to death [3].

The primary defect at the biochemical level that is responsible for the symptoms in the lung was found to involve cAMP-mediated chloride ion (Cl^-) conductance. Specifically, mutations in the gene that encodes a cAMP-regulated Cl^- channel in the apical membrane of epithelial cells are the cause of cystic fibrosis. This gene was identified, cloned and named the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) [4, 5]. Though the exact mechanism of pathogenesis is not fully confirmed, the prevailing theory supports that absence or dramatic decrease in the amount of functional CFTR protein at the airways epithelium results in reduced chloride secretion, increased sodium reabsorption and therefore in insufficient airway luminal fluid due to osmosis [6]. These alterations in the respiratory epithelium subsequently result in deficient mucus clearance which determines chronic cycles of bacterial infections and inflammation [6]. In addition, the formation of thick stationary mucus traps neutrophils that might otherwise clear the infection [7].

For several reasons including the easy access to the respiratory tract without any intervention procedures, the cloning and the characterization of the *CFTR* gene and the expectation that even relatively low levels of expression of the gene may have a therapeutic outcome [8], Cystic Fibrosis became an ideal target for gene therapy and an example for gene therapy of other lung diseases. Indeed, the first gene therapy clinical trials for CF started in 1993 and 29 clinical trials have been conducted since then (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>). Several trials have demonstrated gene transfer and transgene expression. In some cases, low levels of transient correction of Cl^- ion transport deficiency has been observed but overall, no clinical improvement has been achieved. The histological, immunological and intracellular barriers that exist in the lung have proven to be more difficult to overcome than what was initially thought. The purpose of this chapter is to analyze these barriers and to present the challenges the gene therapist is faced with when targeting the lung for the treatment of CF.

First, the basic histology of the lung will be described so that the reader can identify the potential target cells for CF gene therapy and realize the complexity of the lung structures that the gene transfer agent needs to penetrate in order to reach these target cells.

2. Basic histology of the lung

The lung is a complex organ that is divided into the air-conducting portion consisting of the trachea, the bronchi and the bronchioles and the respiratory portion consisting of the alveoli, which is the place of gas exchange (Figure 1).

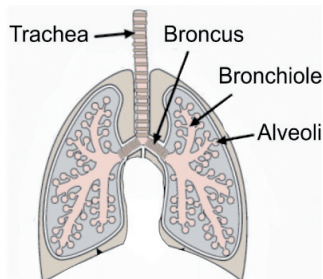


Figure 1. Schematic illustration of the lung.

The trachea and most of the bronchi are covered by pseudostratified columnar ciliated epithelium (known as the respiratory epithelium). Columnar ciliated cells are the predominant population extending from the basal lamina to the airway lumen. Other cells facing the lumen are the nonciliated Goblet cells which produce mucin polymers [9], forming a thin layer of mucus that covers the airway epithelium. The role of the airway mucus is to trap inhaled particles which are then transferred out of the lung by cilia beating and/or cough. The effec-

tiveness of this action depends on the viscosity of the mucus which is determined by the level of its hydration [10]. Normal airway mucus consists of 97% water [11] but when luminal fluid is reduced, as in CF, the clearance of mucus by cilia and cough is also reduced. Under the basal lamina lies the lamina propria, which consists of elastic fibers and hosts the sub-mucosal glands that together with the Goblet cells produce components of the mucus [9] (Figure 2A,B,C).

Several stem cells populations responsible for the maintenance of the respiratory epithelium have been identified in the lung. Specifically, a population of basal cells, residing at close proximity to the underlying basal lamina in the larger airways, has been shown to have a high multipotency potential allowing regulation of the epithelium homeostasis under normal circumstances or after injury [12]. Another type of cells with stem-cell-like properties is the Clara cells. These nonciliated cells are located at the terminal bronchioles and produce a solution similar to the surfactant in the alveoli. Interestingly, Clara cells can multiply and differentiate into ciliated cells to regenerate the bronchiolar epithelium [13].

The respiratory portion of each lung consists of approximately 300 million alveoli. Each alveolus has a thin wall consisting mainly of type I and type II alveolar cells (Figure 2D). Type II alveolar cells are responsible for the secretion of a thin layer of fluid that normally coats the alveolar surface in order to decrease the surface tension at the air-fluid interface, the surfactant. Surfactant turnover is mediated by the phagocytic function of alveolar macrophages, which are also located in the alveolar wall and are frequently seen in the alveolar lumen.

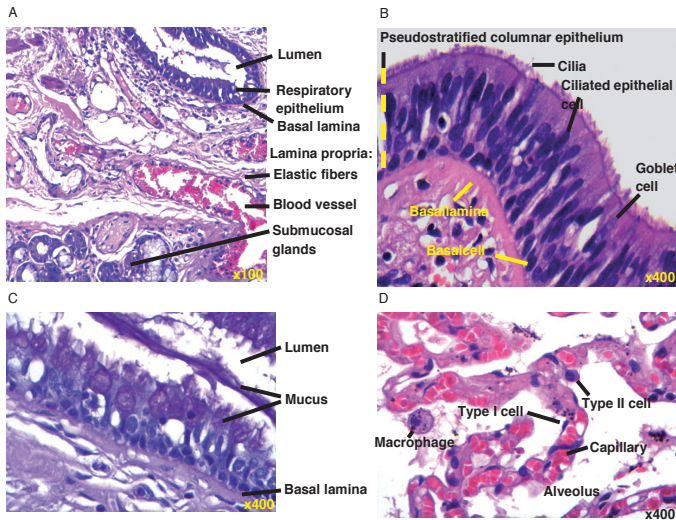


Figure 2. A) Upper respiratory tract section, B) Epithelium of upper respiratory tract, C) Mucus produced in Goblet cells and secreted in the lumen, D) Alveolar section.

It is not yet clear which of these cells are the target for gene therapy of Cystic Fibrosis. In healthy individuals there is little CFTR expression in the lung, except the submucosal glands and epithelial cells at the small airways where higher expression is observed [14]. Early studies suggested that the submucosal glands are the site of maximal CFTR expression [15], but more recent data suggested that this may be the ciliated surface epithelium of the bronchioles [16]. This uncertainty is one of the main obstacles for successful CF gene therapy. Depending on which are the cells that need to be corrected, different anatomical or immunological barriers apply and therefore different administration methods have to be used.

3. Physical barriers to gene transfer to the lung

As most formulations administered to treat CF, including gene transfer agents, are delivered directly to the airways in the form of aerosols [17], the first physical barrier that needs to be overcome before the transgene reaches the target cells is the airways mucus. Trapping in the mucus and clearance by cilia is the main factor reducing transfection efficiency in lung cells of all individuals. In Cystic Fibrosis, the lungs are progressively filled with large amounts of purulent secretions, the sputum, which consists of mucus, DNA, actin, cell debris and inflammatory cells [18]. The most commonly used in gene therapy for CF viral vectors and non-viral liposomal vectors have been proven unable to penetrate the CF sputum [19, 20], suggesting that effective treatment may be achieved only at early stages of the disease before the lungs are filled with sputum [21]. Several improvements can be made at this stage to increase transfection efficiency, such as use of methylcellulose gel formulations to inhibit mucociliary clearance [22] or of mucolytics [23, 24], but these need to be validated in clinical trials.

The second physical barrier to gene transfer to the airway epithelium is the composition of the apical surface of the cells. First, the presence of specific receptors determines the kind of viral vector to be used. For example, adenoviral vectors have low transfection efficiency due to the low abundance of the required receptors on the apical side of most human airway epithelial cells [25] and this is one of the reasons why they are not considered in clinical trials any more [21]. Making the basolateral membrane, which is more abundant in adenoviral receptors [25], accessible to the adenoviral vector has been proposed as an attractive alternative. Indeed, the use of agents such as sodium caprate that can cause transient dissociation of tight junctions, impressively increases transgene delivery and expression in animal models [26, 27] but may not have a clinical application due to the risk of systemic bacterial invasion. Second, the glycocalyx on the apical membrane seems to interfere with the interaction between adenovirus and its few receptors [28]. Removal of sialic acid residues from the glycocalyx by pretreatment with neuraminidase may be an effective way to overcome this physical barrier [28].

Although non-viral vectors are not affected by the problems described above, they are subject to possible destruction by the cell defence mechanisms against foreign DNA invasion.

These mechanisms have evolved to secure the host cell's genetic integrity but in gene therapy they constitute one more hurdle to overcome. Indeed, once the therapeutic DNA enters the target cells in the lung of CF patients, it faces a series of intra-cellular barriers that apply to gene therapy in general and not just to gene therapy for CF. These barriers include degradation by cytosolic nucleases [29, 30] and degradation inside digestive lysosomes formed by transformation of endosomes following endocytosis [31]. Several methods have been implemented so that the transgene can escape the endosomes after internalization. Cationic lipids and polycationic polymers like polyethylenimine (PEI) [32] utilised as chemical vectors in complexes with the transfected DNA, protect it from nucleases and enable it to escape the endosomes. Such complexes carrying the *CFTR* gene have been used to correct the ion transport defect in CF transgenic mice [33] and are currently being tested in clinical practice [34]. Other strategies to protect the therapeutic DNA from the endosomes and therefore to increase the transfection efficiency include the use of pharmacological endosomolytic agents such as chloroquine [35], endosome-disrupting peptides [36-38] and glycerol [39]. All these aim at destabilizing the endosomal membrane so that the contents are released intact to the cytosol but may be of limited clinical value due to safety concerns in the host cells.

The final physical barrier before the *CFTR* transgene enters the nucleus of the non-dividing airway epithelial cells and undergoes transcription is the nuclear envelope [40]. Many viral vectors can efficiently deliver their cargo in the nucleus by exploiting the nuclear transport systems of the host [41], but non-viral vectors are in most cases ineffective in front of the nuclear envelope. Strategies such as the use of chemical vectors based on PEI [42] and of Nuclear Localization Signals (NLS) which are integrated into the transfected DNA and bind to transporter proteins in order to facilitate nuclear entry [43, 44] have been implemented and found to promote nuclear delivery *in vitro*. However, these have not been validated with large therapeutic genes like the *CFTR* and in non-dividing cells *in vivo* and may not be of use for gene therapy for CF [45].

4. Immunological barriers to gene transfer to the lung

Apart from the extracellular and intracellular barriers described above, there is a second line of defence consisting of specific and non-specific immune responses that protect the lung cells against foreign particles which are present in the air. During gene therapy for CF, these immunological mechanisms can be activated by the vector carrying the transgene or the product of the transgene and therefore limit the overall efficacy [46].

Various immunological responses are directed against the carrier of the therapeutic gene before this enters the target cells. Pulmonary macrophages have been shown to ingest adenoviral vectors, but when they were removed before transfection, an increase in transgene expression was observed [47]. Furthermore, humoral immune responses mediated by helper T lymphocytes result in the production of neutralizing antibodies against the vector, which restricts the possibility of re-administration and so, the use of most viral vectors for the treatment of chronic diseases such as CF [46].

Other responses are initiated after the transgene is delivered to the lung cells. Particularly when viral vectors are used, cellular immune responses mediated by cytotoxic T lymphocytes eliminate transduced cells expressing viral proteins resulting in parallel loss of transgene expression [46]. Although in theory non-viral vectors are not associated to such problems as they are less immunogenic than viral vectors, in practice they are usually used in combination with ligands so that they overcome the physical barriers described above and this can provoke immunological reactions similar to those caused by viral vectors [46]. In addition, viral and non-viral vectors can provoke the release of host cytokines which have been shown to inhibit expression of the gene delivered if this is driven by a common viral promoter [48].

Several approaches have been developed to overcome the immunological barriers in the lung. The use of immunosuppressant drugs such as cyclophosphamide have been proved very effective in mice allowing both prolonged transgene expression and repeated administration of an adenoviral vector [49]. Similar results were obtained with corticoid steroids such as dexamethasone [50] and budesonide [51] which were found to decrease inflammation mediated by viral vectors. Other strategies include the co-administration of IL-12 [52] and blockade of CD4⁺ T cells [53-56]. However, all these approaches are likely to cause more damage than benefit, considering that the lungs of CF patients are colonized by pathogenic bacteria, and so they are not applicable in the clinical setting.

On the other hand, non-viral chemical vectors based on cationic lipids can be re-administered without the need to be combined with immunosuppressants [57]. From that aspect, these are safer than viral vectors for gene therapy of CF but still not absolutely harmless as they have been associated with lung toxicity due to provocation of inflammation [58]. Another mediator of inflammation in the lung can be the CpG motifs on the bacterial plasmid DNA which is usually used to clone the therapeutic gene in non-viral gene therapy [59, 60]. Unlike eukaryotic DNA, this dinucleotide is relatively unmethylated in bacteria and can be inflammatory through recognition by toll-like receptor 9 on B cells [61]. As methylation of the CpG motifs prior to gene delivery may decrease the expression of the transgene, the exclusion of any bacteria-derived DNA from the therapeutic construct is a more promising alternative.

5. Safety concerns

Immunological responses elicited by a gene therapy vector do not only pose a barrier to efficient delivery and expression of the therapeutic gene in the target cells but more importantly, they can raise very serious safety issues. This lesson has been learned from a gene therapy human trial where lethal complications were experienced [62]. In that study, an adenoviral vector containing the cDNA of the gene encoding ornithine transcarbamylase (OTC) was administered to 18 patients with partial OTC deficiency, a disease caused by a defect in urea synthesis. The adenoviral vector provoked immunologic and other side effects, such as fever, myalgia and nausea in 17 out of the 18 participants but the 18th patient developed a

serious immune response to the vector that eventually led to his death 98 hours after administration [62]. This death was a setback for all gene therapy clinical studies using viral vectors, as human immune responses cannot be predicted pre-clinically. Apart from the immunological responses caused by the vector, other unfortunate events of different nature have also been found to be associated with reduced safety. Treatment of patients with X-linked severe combined immune deficiency (SCIDX1) using a retroviral vector carrying the γ c gene resulted in the correction of the disease and huge enthusiasm about the future of gene therapy [63]. However, two of the cured patients developed a leukemia-like condition 2-3 years later due to disruption of an endogenous oncogene by integration of the vector [64, 65]. Since vector integration is usually random and uncontrollable, insertional mutagenesis is a general problem that all integrating vectors have. As these problems also apply to most vectors used in gene therapy for Cystic Fibrosis, avoiding unwanted immune response and insertional mutagenesis are two major challenges for the genetic treatment of CF. Strategies to respond to the second challenge of insertional mutagenesis will be discussed in the next section. To address the first problem, the solution is to use less immunogenic vectors.

Although adenoviral vectors administered systemically can cause acute and potentially life-threatening cytokine response [66] their local administration at mild doses to nose and lung tissues did not result in such unacceptable safety profile [34]. However, extensive use of these vectors during the early times of CF gene therapy has shown that cellular and humoral immune responses against the virus are generated and these limit repeated administration [67, 68]. Another approach to avoid immunologic reactions from the host is to coat the virus capsid with polyethylene glycol (PEG). Such PEGylated viruses, called "stealth viruses", are not recognised by the immune system and can significantly prolong transgene expression [69]. A less immunogenic alternative to adenoviral constructs is the use of adeno-associated virus (AAV) vectors. Indeed, several human CF trials with AAV vectors have confirmed their good safety profile albeit with low transduction efficiency [70-74].

Despite significant progress made towards the generation of safer viral vectors [75], non-viral synthetic vectors containing only human DNA sequences are the vectors of choice when safety is considered as first priority. These vectors generally consist of the therapeutic DNA either naked or mixed with chemical compounds, like cationic lipids or cationic polymers [76]. Naked DNA is in theory the safest gene therapy agent but very difficult to be introduced into the target cells. Several physical methods have been developed to facilitate DNA entry into the lung of living animals, such as the use of electrical pulses (electroporation) [77], of ultrasound waves (sonoporation) [78] and of magnetic fields (magnetofection) [79] but none of them has reached the clinical use yet. On the other hand, chemical carriers have rapidly been developed and used in 6% (n=110) of gene therapy clinical trials (<http://www.wiley.com/legacy/wileychi/genmed/clinical>). These act by forming complexes with the negatively charged DNA. The complexes condense the DNA, protect it from nucleases, allow its entry into the cells and protect it from the endosomes [80]. Indeed, local administration of cationic lipid/CFTR-plasmid-DNA complexes in an aerosol formulation to the lungs of cystic fibrosis transgenic mice resulted in correction of the ion transport defect [33]. Simi-

lar studies in human patients demonstrated some transgene expression, but not at sufficient levels to provide a clinical benefit [57, 81-86].

6. Duration of transgene expression

Provided that all obstacles to gene delivery to the lung are overcome and the *CFTR* transgene finally reaches the target cells, a clinical benefit for CF can only be achieved by lifelong expression of the gene. As repeated administration is in most cases restricted by immune responses generated by the patient against the vector, other strategies have been employed for efficient retention and long-term expression.

Integration into the host genome has widely been used in gene therapy to fulfil this requirement. However, the dangers of integration due to insertional mutagenesis have become a widely publicised issue as a result of the SCIDX1 clinical trial, where some patients developed leukaemia due to deregulation of the growth-promoting LIM domain only 2 (LMO2) proto-oncogene caused by integration of the vector [64, 65]. The safety concerns regarding uncontrolled integration of the therapeutic gene into the host genome have been strengthened by observations that there is a preference of integrating vectors for the regulatory regions of transcriptionally active genes [87]. Given the need for long-term expression and the problems associated with vector integration, vectors that persist in the nucleus by being maintained episomally without integrating, could be highly advantageous. Among the systems developed to achieve extra-chromosomal maintenance of the vectors carrying the therapeutic gene, two are considered safe enough for clinical application in the future: artificial chromosomes and systems based on scaffold/matrix attachment region (S/MAR).

Human Artificial Chromosomes (HACs) are vectors able to replicate and segregate in parallel with the endogenous chromosomes in human cells. To achieve this, they must contain the minimal elements required for chromosome function, namely an origin of replication, telomeres and centromeres [88]. HACs can be generated by a method similar to the one applied for YAC construction in yeast and involves assembling the functional chromosomal elements and building up a HAC *de novo* in human cells. Different strategies have been followed to generate *de novo* HACs, the most convenient of which is to transfect a BAC carrying only a large array of α -satellite (alphoid) DNA and some marker genes into HT1080 cells [89]. HACs generated this way exist as single (or low copy) chromosomes in the nucleus and have a high mitotic stability (close to 100%) in the absence of selection. The potential use of these vectors in gene therapy has been demonstrated by expression of large therapeutic genes from them [90, 91].

S/MARs are diverse sequences found in all eukaryotic genomes where they are involved in many aspects of chromatin function such as organization of chromatin into loops, which seems to be mediated by the interaction between S/MARs and the nuclear matrix [92]. Vectors containing an S/MAR element have the ability to remain episomally at low copy number for more than 100 generations in the absence of selection and with a mitotic stability of 98% [93]. This ability has been demonstrated in several cell lines and in primary cells [94]

and also *in vivo* in genetically modified pigs [95] making them very attractive for use in CF clinical trials.

An alternative to the use of episomal vectors described above, that still satisfies both requirements for permanent transgene expression and elimination of genotoxic effects is the controlled integration of the therapeutic DNA at a specific site in the host genome where no active genes are present. Several vector systems have been developed to achieve this, with each one of them having its own limitations [96]. From these, vectors based on the Φ C31 integrase [97] and on transposase enzymes [98] are the most promising for use in CF gene therapy as they have a preference for specific sequences that already exist in the human genome and have been shown to work *in vivo*.

An additional problem to achieving long-term expression of the *CFTR* transgene delivered to the lung is the life span of the target cells. Depending on the rhythm of natural turnover of these cells, transgene expression can last for as long as these cells are alive. A more effective approach would be to either target putative stem cells with the capacity to differentiate to airway epithelial cells [34] or to deliver exogenous heterologous or corrected autologous stem cells to the lungs of CF patients *ex vivo* [99].

Airway basal stem cells are a candidate target for CF stem cell therapy. However, the facts that this population is estimated to represent only a minor part of the total airway epithelium [12] and that it is quite inaccessible as it is not exposed to the airway lumen make such a therapy approach very challenging.

Ex vivo gene therapy using stem cells may not only provide permanent cure without the need for re-administration, but also solve the hurdle of the low *in vivo* gene delivery efficiency. In *ex vivo* cell therapy using Embryonic Stem Cells or foetal Mesenchymal Stem Cells from a healthy embryo there is no need for transfection of a therapeutic gene. However, these stem cells are used in an allogeneic fashion which requires the parallel use of immunosuppressive drugs and are therefore not applicable for the treatment of CF. A more attractive strategy would be the transfer of the *CFTR* gene to patient-derived autologous stem cells such as Mesenchymal Stem Cells (MSCs), which can easily be isolated from the bone marrow or adipose tissue of adults [100] or Induced Pluripotent Stem Cells that can be generated by reprogramming of adult somatic cells [101, 102]. In that case, the transfection procedure would be performed *in vitro* before reimplantation of the cells back to the donor, which is far more efficient than *in vivo* delivery. Furthermore, bone marrow-derived MSCs have been shown to be able to express transgenes [103] and to differentiate to several types of cells including airway epithelial cells [104]. Nevertheless, there is little literature on how the *ex vivo* corrected MSCs can be administered and engrafted in the lung of Cystic Fibrosis patients. Both systemic and topical lung administration of bone marrow-derived cells have been applied and shown to result in some engraftment into the airways [105], but there are several challenges to be addressed, before *ex vivo* cell therapy becomes part of the CF clinical research. These challenges include the very low efficiency of engraftment (<1%) and the fact that previous damage to the surface epithelium caused by epithelia-injuring reagents seems to be required for the engraftment [75].

7. Pattern of transgene expression

For gene therapy of some diseases it is important to achieve expression of the therapeutic gene at specific levels. Expression at lower levels than normal might not be sufficient to correct the defect and at higher levels could result in undesirable effects. In other cases, tissue-specific expression may be very important. The elements responsible for controlled and tissue-specific expression of a gene usually lie within the introns and the sequences before and after the gene. Therefore, the use of genomic constructs which contain the introns and flanking DNA of the therapeutic gene is expected to be more effective than that of mini-gene/cDNA constructs in gene therapy for certain genetic diseases where precise levels of the gene product are required [88]. There is evidence that CF is such a disease.

Although some studies have shown that expression of as little as 5-10% of endogenous *CFTR* levels may suffice to observe a clinical benefit [106], other studies have shown that different functions of *CFTR* like Cl^- transport and Na^+ absorption, when they are abnormal they can be restored by different levels of *CFTR* expression [107]. Moreover, restoration of mucus transport at normal rates requires transduction of at least 25% of target cells [108]. These data indicate that CF gene therapy may require *CFTR* expression at the right levels, at the right time and in the right population of cells, which can be achieved only if it is driven and controlled by the gene's natural promoter and regulatory elements present on a genomic therapeutic construct.

The *CFTR* gene is located on chromosome 7, is 200-250 kb long [5] and comprises 27 exons. It shows a tightly regulated temporal and spatial pattern of expression [109, 110], which was not found to be regulated by any tissue-specific regulatory elements, suggesting that other elements outside the proximal promoter are probably involved in tissue specific regulation of transcription. Several DNase I Hypersensitive Sites (DHS), usually associated with regulation of transcription, have been identified across 400 kb of DNA flanking the *CFTR* gene. These lie 5' to the gene at -79.5 and -20.9 kb with respect to the translation start site [111], in introns 1 [112], 2, 3, 10, 16, 17a, 18, 20 and 21 [113] and 3' to the gene at +5.4, +6.8, +7, +7.4 and +15.6 kb [114]. Most of these DHS have been found to be involved in tissue-specific *CFTR* expression [114-117]. Therefore, a large genomic construct spanning ~300 kb from the -79.5 kb to the +15.6 kb DHS would include all the known long-range controlling elements of the *CFTR* gene and should give full levels of tissue specific expression which would be advantageous for gene therapy of cystic fibrosis. This big in size region has recently been cloned on a single Bacterial Artificial Chromosome (BAC) vector and is currently available [118].

As the majority of recombinant viruses, commonly utilized as carriers for transfer of plasmid DNA, apart from evoking unwanted immune responses, have a maximum packaging capacity and cannot be used to deliver large genomic-DNA-containing constructs, gene therapy using genomic loci of therapeutic genes should be non-viral. This restriction raises again the issue of efficiency of delivery which is even more challenging to deal with than when using smaller constructs.

8. Clinical research for cystic fibrosis

Gene therapy clinical trials for CF started in 1993 and over 26 viral and non-viral trials have been conducted or are in progress to date. Viral trials were based on engineered adenovirus and adeno-associated virus and non-viral on various cationic lipids, with GL67 being the most predominant. The references for the published studies are listed in Table 1.

Adenoviral clinical trials	AAV clinical trials	Non-viral clinical trials using cationic lipids
Zabner <i>et. al.</i> , 1993 [119]	Wagner <i>et. al.</i> , 1999 [120]	Sorcher <i>et. al.</i> , 1994 [121]
Crystal <i>et. al.</i> , 1994 [122]	Aitken <i>et. al.</i> , 2001 [70]	Caplen <i>et. al.</i> , 1995 [82]
Boucher <i>et. al.</i> , 1994 [123]	Wagner <i>et. al.</i> , 2002 [74]	Gill <i>et. al.</i> , 1997 [83]
Knowles <i>et. al.</i> , 1995 [124]	Flotte <i>et. al.</i> , 2003 [71]	Porteous <i>et. al.</i> , 1997 [84]
Hay <i>et. al.</i> , 1995 [125]	Moss <i>et. al.</i> , 2004 [73]	Zabner <i>et. al.</i> , 1997 [86]
Zabner <i>et. al.</i> , 1996 [68]	Moss <i>et. al.</i> , 2007 [72]	Alton <i>et. al.</i> , 1999 [81]
Bellon <i>et. al.</i> , 1997 [126]		Hyde <i>et. al.</i> , 2000 [57]
Harvey <i>et. al.</i> , 1999 [67]		Noone <i>et. al.</i> , 2000 [127]
Zuckerman <i>et. al.</i> , 1999 [128]		Ruiz <i>et. al.</i> , 2001 [85]
Joseph <i>et. al.</i> , 2001 [129]		
Perricone <i>et. al.</i> , 2001 [130]		

Table 1. List of CF gene therapy clinical trials.

The trials have confirmed several of the safety concerns associated to the use of viral vectors. However, other challenges and questions raised pre-clinically still remain to be answered. In general, proof-of-principle for gene transfer to the airways has been demonstrated by transgene expression or partial correction of the CF transport defect in some of the trials but clinically meaningful outcomes such as improvement in pulmonary function and decrease of bacterial colonies have not been clearly shown in any. In contrast, poor results with regards to clinical benefit, obtained so far, revealed another challenge in CF clinical research. This is the need to develop more accurate tools to assess gene transfer efficacy at the clinical level [34].

Historically, adenoviral vectors were the first to be used. However, due to the absence of adenoviral receptors on the apical side of most human airway epithelial cells [25], which results in low transduction efficiency, and due to induction of immune responses that exclude repeated administration [67, 68], adeno-associated viral vectors became an alternative. These vectors were soon found to have their own problems. First it is their small packaging capacity which barely holds the whole human *CFTR* gene and therefore restricts the use of strong promoters. Then, at least serotype 2 AAV vectors that were used in initial studies could not be re-administered due to stimulation of immune reactions [72, 73]. Suggestions made to

overcome these limitations still need to be validated in humans. Non-viral vectors were found compatible with repeated administration [57], but their efficiency were variable and transgene expression was shown only in some studies. In addition, flu-like symptoms were reported [81, 85], which were associated to the presence of unmethylated CpG motifs on the plasmid DNA that was delivered. The use of genomic constructs containing only human DNA may overcome this limitation but this also needs to be shown in future clinical trials.

9. Conclusion

Almost 20 years have passed since the beginning of gene therapy trials for CF. Despite initial enthusiasm, only little progress has been made during that time. In contrast, the main conclusion was that the lung is more difficult to target than initially anticipated. Several barriers were discovered, which led to the development of respective ways to overcome them. The majority of these have not reached the level of validation in clinical trials yet. For example, the use of a non-viral vector with the ability to remain extra-chromosomally containing the whole genomic region of the *CFTR* gene or *ex vivo* stem cell therapy, are two promising approaches that need to be further explored and may be seen in clinical trials in the future.

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Gene Therapy for the *COL7A1* Gene

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Epidermolysis bullosa

Epidermolysis bullosa (EB) is a genetically and clinically variable disease characterized by blister formation and erosions of the skin and mucous membranes after minor trauma [1]. The inheritance of the affected genes can occur in a dominant or recessive way depending on the subform of the disease. In general, epidermolysis bullosa is caused by mutations in genes encoding structural proteins within the basal membrane zone of the skin. Absence or functional loss of one of these proteins results in a lack of stability of the microarchitecture of the connection between dermis and epidermis leading to a loss of coherence [1]. The basement membrane between the dermis and the epidermis is a complex membrane produced by basal keratinocytes and dermal fibroblasts that acts as mechanical support for the connection of both skin layers. The basal membrane also regulates the metabolic exchange between the two skin compartments [2]. Up to date, there are at least 15 genes associated with EB causing different forms of the disease. Numerous mutations in these genes that encode for structural proteins within keratinocytes or within mucocutaneous basement membranes have been identified up to now [1].

Mutations in the genes, encoding for the keratins 5 and 14 and plectin, lead to epidermolysis bullosa simplex (EBS) characterized by the cytolysis within basal keratinocytes. Junctional epidermolysis bullosa (JEB) is caused by the absence or loss of function of laminin-332, type XVII collagen or integrin- $\beta 4$. JEB is a severe EB form and is characterized by the separation of the skin within the lamina lucida. Mutations in type VII collagen (encoded by *COL7A1*) lead to the dystrophic form of epidermolysis bullosa, characterized by skin separation below the lamina densa. The severity and clinical manifestation of the disease depend on the mutation type (missense mutation, nonsense mutation, splice site mutations, deletion or insertion), the mode of inheritance and the localization of the mutation within the gene. Due to this fact, diagnosis,

course of disease and therapy vary significantly depending on the present EB subform [3]. Blister formation can be restricted to the soles of the feet or occur generalized. Severe systemic complications and extracutaneous manifestations including blistering and erosions of the cornea and mucosal tissues, stenoses or strictures of respiratory, gastrointestinal and urogenital tracts, pylorus atresia, muscular dystrophy and skin cancer are certain complications associated with different EB subtypes [1]. So far over 30 distinctive subtypes have been described and classified in a system, which was recently revised [4]. See Table 1.

A: Classification scheme for the major EB subtypes		
Major EB type	Major EB subtypes	Affected proteins
EB simplex (EBS)	Suprabasal EBS	plakophilin-1, desmoplakin;
		others?
	Basal EBS	keratins 5 & 14; plectin, α6β4 integrin, BPAG1
Junctional EB (JEB)	JEB, Herlitz (JEB-H)	laminin-332, (laminin-5)
	JEB, other	laminin-332, type XVII collagen
		α6β4 integrin, α3 integrin
Dystrophic EB (DEB)	Dominant DEB (DDEB)	type VII collagen
	Recessive DEB (RDEB)	type VII collagen
Kindler syndrome		kindlin-1
B: Classification scheme for all known EB simplex subtypes		
Major types	EBS subtypes	Affected proteins
EBS suprabasal	<i>lethal acantholytic EB</i>	desmoplakin
	<i>plakophilin deficiency</i>	plakophilin-1
	<i>EBS superficialis</i>	?
EBS basal	EBS, localized (EBS-loc) ^a	K5, K14
	EBS, Dowling Meara (EBS-DM)	K5, K14
	EBS, other generalized (EBS,gen-nonDM) ^b	K5, K14, BPAG1
	<i>EBS with mottled pigmentation(EBS-MP)</i>	K5
	EBS with muscular dystrophy (EBS-MD)	plectin
	<i>EBS with pylorus atresia (EBS-PA)</i>	plectin, α6β4 integrin
	<i>EBS, autosomal recessive (EBS-AR)</i>	K14
	<i>EBS, ogna (EBS-Og)</i>	plectin
	<i>EBS, migratory circinate (EBS-migr)</i>	K5
<i>(rare variants in italics)</i>		
^a Previously called EBS, Weber-Cockayne		
^b Includes patients previously classified as EBS-Koebner		
C: Classification scheme for all known junctional subtypes		
Major JEB subtype	Subtypes	Affected proteins
JEB, Herlitz (JEB-H)		laminin-332
JEB, other (JEB-O)	JEB, non-Herlitz, generalized (JEB-nH gen) ^a	laminin-332, type XVII collagen

	JEB, non-Herlitz localized (JEB-nH loc)	typeXVII collagen
	JEB with pyloric atresia (JEB-PA)	$\alpha 6\beta 4$ integrin
	<i>JEB, inversa (JEB-I)</i>	laminin-332
	<i>JEB, late onset (JEB-lo)^a</i>	
	<i>LOC syndrome (laryngo-onycho-cutaneous syndrome)</i>	laminin-332 $\alpha 3$ chain
	?	$\alpha 3$ integrin
<i>(rare variants in italics)</i>		
^a Formerly known as generalized atrophic benign EB (GABEB)		
^b Formerly known as EB progressive		
D: Classification scheme for all known dystrophic EB subtypes		
Major DEB subtype	Subtypes	Affected protein
DDEB	DDEB, generalized (DDEB-gen)	type VII collagen
	<i>DDEB, acral (DDEB-ac)</i>	
	<i>DDEB, pretibial (DDEB-Pt)</i>	
	<i>DDEB, pruriginosa (DDEB-Pr)</i>	
	<i>DDEB, nails only (DDEB-no)</i>	
	<i>DDEB, bullous dermolysis of the newborn (DDEB-BDN)</i>	
RDEB	RDEB, severe generalized (RDEB-sev gen) ^a	type VII collagen
	RDEB, generalized other (RDEB-O)	
	<i>RDEB, inversa (RDEB-I)</i>	
	<i>RDEB, pretibial (RDEB-Pt)</i>	
	<i>RDEB pruriginosa (RDEB-Pr)</i>	
	<i>RDEB, centripetalis (RDEB-Ce)</i>	
	<i>RDEB, bullous dermolysis of the Newborn (RDEB-BDN)</i>	
<i>(rare variants in italics)</i>		
^a Previously called RDEB, Hallopeau-Siemens		

Table 1. Classification system for inherited epidermolysis bullosa. Based on Fine et al. [4].

2. Dystrophic epidermolysis bullosa (DEB)

Mutations in the gene COL7A1, encoding for type VII collagen, cause the dystrophic form of epidermolysis bullosa (DEB). Type VII collagen is the major constituent of the basement membrane’s anchoring fibrils and belongs to the superfamily of collagens [5]. COL7A1 comprises 118 exons and mostly short intervening introns resulting in a size of the entire COL7A1 gene of 32kb encoding an mRNA of over 9kb [6,7]. The remarkable number of COL7A1 mutations and the variable genotype-phenotype correlation hamper the finding of an optimal therapy for DEB patients. Nevertheless severity of clinical manifestations can often be defined by the type of the mutation and its localization within the COL7A1 gene [3]. DEB is divided into two main subtypes according to the mode of inheritance. Dominant dystrophic EB (DDEB) is inherited in an autosomal dominant way, whereas recessive dystrophic EB (RDEB) is transmitted in an autosomal recessive mode [8]. RDEB is classified in

severe generalized RDEB (RDEB-sev gen) – formerly called RDEB, Hallopeau Siemens - and RDEB-generalized other (RDEB-O) – formerly called RDEB-non Hallopeau Siemens [3].

The DDEB phenotype is mostly generalized but mild and clinically characterized by recurrent blistering, milia, atrophic scarring, nail dystrophy and eventual loss of nails [3]. See Figure 1A,B. The fact that the defective and wildtype alleles are expressed equally explains the relative mild phenotype in comparison to RDEB [3]. Missense mutations or in frame deletions in *COL7A1* causing RDEB disturb the assembly and aggregation of type VII collagen into anchoring fibrils. As a result, the number of anchoring fibrils and their morphology is altered significantly. The resulting subforms of RDEB are classified as RDEB, generalized other [3]. RDEB-sev gen is caused by nonsense mutations in both alleles, resulting in a complete loss of type VII collagen within the basal membrane zone of the skin. Clinical manifestations of RDEB are generalized blistering, erosions, crusts, atrophic scarring, onychodystrophy, loss of nails, mutilating pseudosyndactyly of hands and feet and functionally disabling contractures in hands, feet, elbows and knees. See Figure 1C-H. Additionally, severe extracutaneous complications as gastrointestinal and urogenital tracts involvement, external eye, chronic anaemia, growth retardation and a high risk for the development of aggressive squamous cell carcinoma decrease (Figure 1 I) the life quality of the patient [3,9-14].



Figure 1. Clinical phenotype of EB. **A,B:** DDEB with milia formation and atrophy. **C:** Atrophic scar with crusts and erosions in RDEB. **D:** Boy with severe-generalized RDEB leading to ulcerations and large non healing wounds with atrophic scarring at the back; **E:** Nail dystrophy on both feet. **F,G:** Mitten formation in hands and feet. **H:** Severe caries **I:** Squamous cell carcinoma on the foot (Photos: R. Hametner)

3. The dermal epidermal junction

The blisters characteristic for EB arise within the dermal-epidermal junction. Having a look at this compartment of the skin helps to understand the cause of blistering in EB. The dermal-epidermal junction is a complex basement membrane synthesized by dermal fibroblasts and basal keratinocytes. Adhesion of the epidermis to the underlying dermis is mechanical-

ly supported by the so called basement membrane zone (BMZ). Moreover it regulates the metabolic exchange between these two compartments. Up to now more than 20 macromolecules situated in the dermal-epidermal-junction have been detected and characterized at biochemical and genomic level [2].

Three protein-junction complexes stabilize the adherence of the basal keratinocytes to the dermis. See Figure 2. The hemidesmosomes built up by plectin, the bullous pemphigoid antigen 1 (BPAG1), $\alpha 6\beta 4$ integrin and type XVII collagen (bullous pemphigoid antigen 2 - BPAG2) link the basal keratinocytes with the basement membrane, spanning the lamina lucida and anchored in the lamina densa [2]. Different laminin isoforms are located in the lamina lucida (laminin-332, laminin 6, laminin 10) and contribute along with BPAG2 to the formation of the anchoring filaments. The lamina densa is mainly built up by type VII collagen anchoring the lamina densa to the underlying dermis by the formation of anchoring fibrils [2]. Some other antigens as uncein (19-DEJ-1 antigen), NU-T2 antigen, KF1 antigen, LDA1 antigen, nidogen, heparin-sulfate, proteoglycan, antigens AF1 and AF2, thrombospondin, type V collagen and osteonectin/BM-40 have been detected in the lamina densa but have not yet been adequately characterized [2].

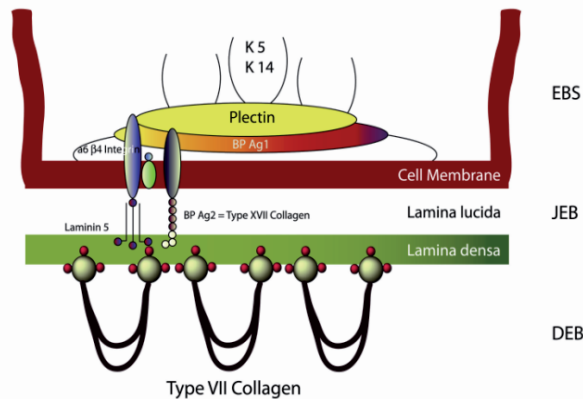


Figure 2. Schematic setup of the cutaneous dermal-epidermal junction zone and localization of structural proteins affected in inherited EB (Diagram by R. Hametner). laminin 5 = laminin 332; EBS = epidermolysis bullosa simplex; JEB = junctional epidermolysis bullosa; DEB = dystrophic epidermolysis bullosa

4. Type VII collagen

Type VII collagen is classified in the superfamily of collagens [7]. A protein domain in triple-helical conformation, which provides stability and integrity between connective tissues, is a common structural feature of all collagens [7]. Type VII collagen is a minor collagen in human

skin and demonstrates spatially restricted location but it plays a critical role in providing integral stability to the skin because it is the major component of the anchoring fibrils [6,7].

5. Biology of type VII collagen

Type VII collagen molecules are characterized by the two non-collagenous NC-1 and NC-2 domains flanking a central collagenous, triple-helical segment [7]. In contrast to other interstitial collagens the repeating Gly-X-Y collagenous sequence is interrupted by 19 imperfections due to insertions or deletions of amino acids. There is a 39 amino acid non-collagenous hinge region susceptible to proteolytic digestion with pepsin in the middle of the triple-helical domain [15]. The amino terminal NC-1 domain (approximately 145kD in size), is built up of sub-modules with homology to known adhesive proteins, including segments with homology to cartilage matrix protein (CMP), nine consecutive fibronectin type III-like (FN-III) domains, a segment with homology to the A domain of von Willebrand factor, and a short cysteine and proline-rich region [15]. The C-terminal non-collagenous NC-2 domain is with 30kD in size relatively small, and contains a segment with homology to Kuniz protease inhibitor molecule [16,17].

The 32kb gene encoding a 9,2kb mRNA has been mapped to the short-arm of chromosome 3p21.1 [18]. The encoding primary sequence and the gene structure of type VII collagen are well conserved. The mouse gene shows 90.4% identity at the protein level and 84.7% homology at the nucleotide level, indicating the importance of type VII collagen as a structural protein [19].

The expression pattern of *COL7A1* is tissue specific and restricted. Type VII collagen has been detected by immunomapping to a selected number of epithelia, including the dermal-epidermal BMZ of skin, the amniotic epithelial BMZ of the chorioamnion, the corneal epithelial basement membrane (Bowman's membrane) and the epithelial basement membrane of oral mucosa and cervix. Moreover the presence of type VII collagen correlates with the presence of ultrastructurally detected anchoring fibrils [6]. A number of cytokines modulate type VII collagen expression. Especially transforming growth factor- β is a powerful upregulator of *COL7A1* at transcription level in fibroblasts and keratinocytes [20,21].

6. Type VII collagen – A major component of the anchoring fibrils

Type VII collagen is synthesized by two cell types in the skin: keratinocytes and fibroblasts [22]. After synthesis of complete pro- α 1 (VII) polypeptides, three polypeptides are associated through their carboxy-terminal ends to a trimer molecule, which is then folded in its collagenous segment into the triple-helical formation. Past to secretion into the extracellular milieu two type VII collagen molecules are aligned into an anti-parallel dimer with the amino-terminal domains present at both ends of the molecule [6]. During dimer-assembly stabilization by inter-molecular disulfide bond formation and a proteolytic removal of a part of the carboxy-terminal ends (NC-2 domain) of both type VII collagen molecules take place [23]. Large num-

bers of these anti-parallel dimers aggregate laterally to form anchoring fibrils, which then can be identified by their characteristic, centro-symmetric banding patterns in transmission electron microscopy [7].

The affinity of the NC-1 domain to bind the principal components of the cutaneous basement membrane, laminin-332, laminin-311 and type IV collagen provides stability to the dermo-epidermal adhesion on the dermal site at the lamina lucida/papillary dermis interface [6,24,25]. Arg-Gly-Asp sequences in the NC-1 domain serve as integrin mediated attachment sites for cells to adhere to extracellular matrix components such as fibronectin [26].

7. Mutations in COL7A1

Mutations in *COL7A1* have clinical consequences in terms of disrupted integrity of the skin, due to the complexity of the *COL7A1* gene, type VII collagen protein structures and the critical importance of its distinct domains in macromolecular interactions [7]. At least 324 pathogenic mutations have been detected within *COL7A1* in different variants of DEB up to now including 43 nonsense, 127 missense, 65 deletion, 28 insertion, 9 insertion-deletion, 51 splice-site and 1 regulatory mutations [27]. See Figure 3-5. Exon 73 constitutes a region with a high frequency of mutations, what suggests being a region in which mutations commonly affect the function of anchoring fibrils [28]. RDEB is caused by nonsense, splice-site, deletions or insertions, silent glycine substitutions within the triple helix and non-glycine missense mutations within the triple-helix or non-collagenous NC-2 domain [29]. RDEB-severe generalized originates from nonsense, frameshift or splice-site mutations on both alleles leading to premature termination codons (PTCs) [30], which result in nonsense mediated mRNA decay or truncated proteins, leading to a reduced number of collagen VII monomers, which are unable to assemble into functional anchoring fibrils [29,30]. PTC mutations do not cause a clinical phenotype if they appear in the heterozygous state, but if they are homozygous or combined with another PTC mutation they are causing severe generalized RDEB [27]. Two missense mutations or compound heterozygosity of a missense and a PTC mutation lead to severe generalized RDEB in very rare cases [31].

RDEB, generalized other, the milder phenotype, is mostly caused by PTCs, small deletions, substitutions of glycine residues in the collagenous domain, splice-site mutations within NC-2 [32-35], delayed termination codons [36], in frame exon skipping [29,36], or missense substitution mutations involving amino acids other than glycine [29,37,38], the majority involving arginine residues resulting either in the loss of an ionic charge or in the introduction of a bulky chain at an external position of the triple helix [27]. Thereby these mutations usually concern a critical amino acid and change the conformation of the protein, which then might still be able to assemble into a small number of anchoring fibrils but is likely to be unstable when they laterally aggregate. Anyhow some full length type VII collagen polypeptides can still be built up [39].

DDEB is caused by glycine substitutions within the triple helical domain of *COL7A1* or other missense mutations, deletions or splice site mutations in some cases [5,26,40-44]. Critical ami-

no acids in the structure of the triple helix are affected by these mutations and therefore the overall stability of the anchoring fibrils is disturbed. More than 100 missense mutations resulting in a Gly-Xaa substitution have been detected in the collagenous domain of COL7A1; half of these are situated in amino acids 1522-2791 and have a dominant negative effect [27].

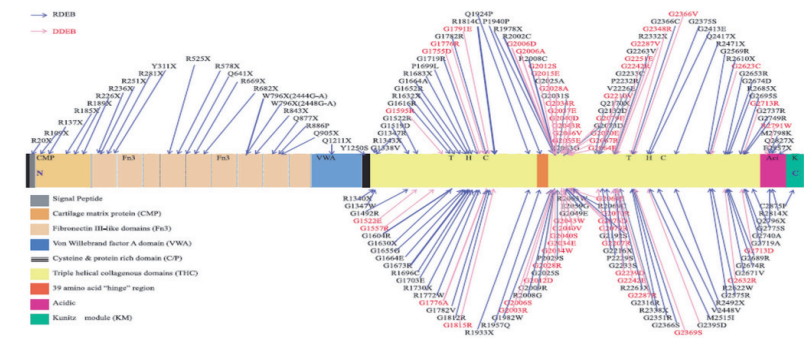


Figure 3. Missense and nonsense mutations in DEB patients. The red lettering signifies dominant and the black signifies recessive inheritance. (Dang et al. [27] © 2008 Blackwell Munksgaard, Experimental Dermatology)

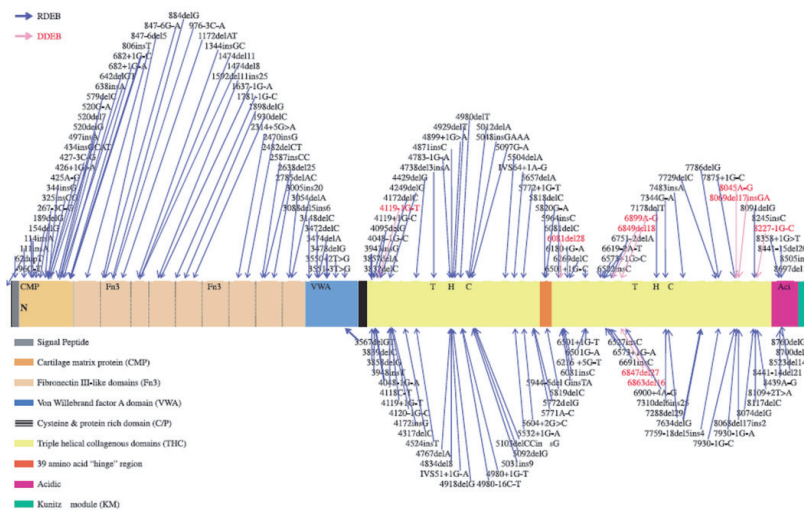


Figure 4. COL7A1 deletions, insertions and splice site mutations in DEB patients. The red lettering signifies dominant and the black signifies recessive inheritance. (Dang et al. [27] © 2008 Blackwell Munksgaard, Experimental Dermatology)

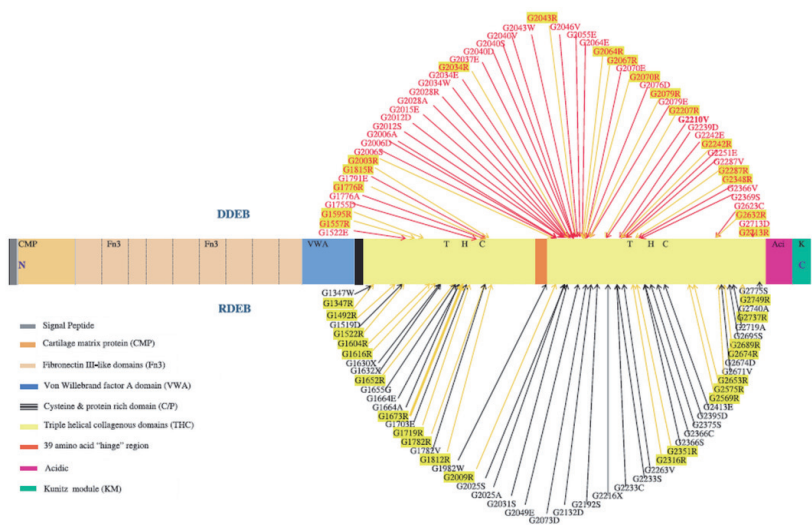


Figure 5. Glycine substitutions in DEB. These are all in the triple-helical collagenous domain; the ones above represent DDEB, the ones below RDEB. (Dang et al. [27] © 2008 Blackwell Munksgaard, Experimental Dermatology)

8. Mouse model

So far there are only two viable mouse models with defects in the COL7A1 gene. A transgenic mouse carrying human COL7A1 cDNA inclusive the human 7528delG mutation in exon 101, which develops the DEB phenotype gradually [45], and a collagen VII hypomorphic mouse published by Fritsch et al. 2008 [46]. In the collagen VII hypomorphic mouse reduced expression of collagen VII originates from aberrant splicing resulting from the introduction of a phosphoglycerate kinase promoter-driven neomycin phosphotransferase expression cassette (PGK-Neo cassette) in intron 2 of COL7A1. One out of three possible splice variants is translated into full-length type VII collagen resulting in a reduction of type VII collagen levels to about 9% of wildtype levels in COL7A1^{flNeo/flNeo} mice. Hemorrhagic blisters on the soles of fore and hind paws, ears and mouth are developed by the collagen VII mice within the first 48 hours of life. Blisters of newborn COL7A1^{flNeo/flNeo} mice were histopathologically classified as hemorrhagic and subepidermal. Type VII collagen immunofluorescence staining revealed weak reactivity in comparison to wildtype littermates. Ultrastructurally normal but reduced in number anchoring fibrils were detected in transmission electron microscopy of the dermal-epidermal junction of the skin. COL7A1^{flNeo/flNeo} mice suffer from growth retardation due to malnutrition and subsequently have a reduced life expectancy. Moreover healing of the initial blistering on the paws with scarring results in the development of mitten deformities beginning at 2-3 weeks of age [46].

9. Therapy approaches

Due to the size of the *COL7A1* gene, a causal therapy for dystrophic epidermolysis bullosa is a great challenge. Symptomatic therapy is concentrated on prevention of skin trauma to minimize blister formation, prevention of secondary bacterial infection, treatment of infection, measures to improve wound healing, maintenance of good nutrition, treatment of correctable complications, and finally rehabilitation [3]. However, several gene and cell therapy strategies showed the potential to revert the disease-associated phenotype. Phenotypic correction of recessive DEB forms (RDEB) can be achieved by gene insertion therapy, in which the wildtype sequence of a mutated gene of interest is introduced into the target cells. Moreover alternative avenues including gene-, cell-, protein- and other systemic- therapy approaches have been tested to restore type VII collagen expression. See Table 2.

Author	Approach	Year
Woodley et al.	Type VII collagen minigene	2000
Sat et al.	Cosmid clone containing the entire <i>COL7A1</i> gene	2000
Mecklenbeck et al.	Microinjection of a <i>COL7A1</i> -PAC vector	2002
Urda et al.	ΦC31 bacteriophage integrase	2002
Chen et al.	Minimal lentiviral vectors	2002
Baldeschi et al.	Canine type VII collagen	2003
Woodley et al.	Targeting fibroblasts instead of keratinocytes (lentivirally)	2003
Gache et al.	Full-length cDNA (retrovirally)	2004
Woodley et al.	Intradermal injection of recombinant type VII collagen	2004
Woodley et al.	Intradermal injection of lentiviral vectors in vivo	2006
Goto et al.	Targeting fibroblasts instead of keratinocytes (retrovirally)	2006
Goto et al.	Targeted exon skipping using antisense	2006
Wong et al.	Intradermal injection of allogenic wildtype fibroblasts into a patient	2007
Fritsch et al.	Intradermal injection of murine wildtype fibroblasts in a DEB mouse model	2008
Remington et al.	Intradermal injection of human type VII collagen in mice	2009
Titeux et al.	Minimal self-inactivating retroviral vectors harbouring the full length human <i>COL7A1</i> gene	2010
Wagner et al.	Allogeneic bone marrow transplantation	2010
Siprashvili et al.	Full-length cDNA (retrovirally)	2010
Muraier et al.	3' Trans-splicing of <i>COL7A1</i>	2011

Table 2. Therapy approaches to restore type VII collagen expression

Woodley et al. used a type VII collagen minigene, which contains the intact noncollagenous domains NC1 and NC2 and part of the central collagenous domain. This approach resulted after transduction into DEB keratinocytes in persistent synthesis and secretion of a 230kDa recombinant minicollagen VII [47]. However deletions in *COL7A1* have been reported to be associated with a pathologic phenotype [5,48,49]. The same group introduced recombinant human type VII collagen into mouse and human skin equivalents transplanted onto mice, by injection. As a result the injected type VII collagen was detected within the basal membrane zone leading to a reversion of the disease associated phenotype [50]. Additionally, the group expressed type VII collagen using a self-inactivating lentiviral vector, which was injected into human skin equivalents, expanded from DEB cells, placed on nude immunodeficient mice. Experiments revealed the synthesis and insertion of the protein into the basal membrane zone [51]. Using a cosmid clone, carrying the entire *COL7A1* gene, was also shown to be a promising way to direct expression of type VII collagen in skin in fetal and neonatal mice. The tested neonatal or fetal mice produced type VII collagen within the basal membrane zone of the skin showing a stable expression of the protein *in vivo* [52]. Microinjection of a P1-derived artificial chromosome (PAC) carrying the entire *COL7A1* locus resulted in production of a procollagen VII similar to the authentic one by Mecklenbeck et al. [53]. The Φ C31 bacteriophage integrase, facilitating integration only in pseudo attP sites, was used to integrate *COL7A1* stably into DEB primary epidermal progenitor cells by Urda et al. [54]. Baldeschi et al. also showed sustained and permanent expression of the transgene after transduction of canine type VII collagen into human and canine DEB keratinocytes [55]. Based on this study Gache et al. yield a full phenotypic reversion of the disease-associated phenotype of RDEB epidermal clonogenic cells after full-length human *COL7A1* cDNA introduction using a retroviral system. However, the expression of *COL7A1* was 50 times higher than the levels monitored in wildtype keratinocytes in monolayers, increasing the risk for an ectopic transgene expression and an abnormal accumulation in skin equivalents [56]. Chen et al. used a minimal lentiviral vector for *COL7A1* expression *in vitro* as an alternative to the retroviral system applied by Gache et al. [57]. Goto et al. showed that *COL7A1* treated fibroblasts of skin grafts provide higher amounts of type VII collagen for the dermal-epidermal junction than keratinocytes [58]. They have also demonstrated an antisense oligoribonucleotide therapy to maintain exon skipping of an exon comprising a premature stop codon. As a result a truncated type VII collagen variant was expressed [59]. Additionally, intradermal injection of untreated normal human or gene-corrected fibroblasts in mice can result in a stable production of human type VII collagen at the basal membrane zone of the skin [60]. Moreover, Wong et al. demonstrated an increased source of type VII collagen in the dermal-epidermal junction for at least three months after intradermal injection of allogeneic fibroblasts [61]. In a mouse model Fritsch et al. showed an accumulation of type VII collagen and restoration of a functional dermal-epidermal junction after injection of murine wildtype fibroblasts into a type VII collagen hypomorphic mouse [46]. In 2009 Remington et al. injected human type VII collagen into *COL7A1* $-/-$ mice, also restoring type VII collagen expression and correct generation of anchoring fibrils [62]. Titeux et al. transduced *COL7A1* cDNA under the

control of a human promoter using a minimal self-inactivating retroviral vector into RDEB keratinocytes and fibroblasts leading to cell correction and long lasting expression of type VII collagen. The dermal-epidermal junction in generated skin equivalents was restored [63]. A similar strategy was shown by Siprashvili et al. using an epitope-tagged *COL7A1* cDNA, providing a long term expression of the protein in skin equivalents [64]. In a clinical trial executing a bone marrow transplantation 6 RDEB patients received allogeneic stem cells to milder the RDEB phenotype. As a result, 5 patients showed an improved wound healing, but one patient died [65].

Until now, no *ex vivo* gene therapy approach passed through a phase I/II gene therapy trial. Most of these applications are focusing on the transfer of full-length *COL7A1* cDNA into the affected patient cells. The drawbacks of the insertion of the full-length 9kb cDNA of *COL7A1* are the cloning and packaging limitations of commonly used vector systems to transduce keratinocytes or fibroblasts and the instability of the *COL7A1* gene due to possible genetic rearrangements of the large repetitive cDNA sequence [47]. Additionally, the influence of *COL7A1* over- or ectopic expression in treated cells has to be clarified for a clinical application. Using the methodology of spliceosome mediated RNA *Trans*-splicing (SMaRT) can be a promising alternative to the mentioned approaches to cope with some of the suspected issues present in full-length *COL7A1* replacement strategies. Murauer et al. demonstrated the exchange of the 3' coding *COL7A1* cDNA region spanning from exon 65 to the last exon 118 by SMaRT [66]. Thereby the risk of genetic rearrangements of the *COL7A1* cDNA sequence should be reduced significantly. Alternatively to this, we will present in this work a 5' exon replacement strategy using SMaRT, providing the possibility to repair also relevant mutations 5' within the *COL7A1* gene.

10. Spliceosome mediated mRNA *Trans*-splicing

10.1. General aspects

RNA *trans*-splicing is a naturally occurring event to recombine two or more mRNA molecules to a new chimeric gene product [67]. For therapeutic purposes such products can be generated by *trans*-splicing a second RNA species from a RNA *trans*-splicing molecule (RTM) into the 3', 5' or internal sequence of an endogenously expressed target. See Figure 6. The main advantages of this methodology are the possibility to reduce the size of the transgene, the maintained endogenous regulation of transgene expression and the feasibility to treat dominant negative diseases [68]. Undesired gene expression due to unintended delivery or misregulation is minimized as *trans*-splicing should only occur in cells expressing the target pre-mRNA [69]. Furthermore, SMaRT offers the potential for correction of dominant negative mutations into wildtype gene products [70].

10.2. Methodology of spliceosome mediated mRNA *trans*-splicing (SMaRT)

In SMaRT constructs that are engineered to bind the introns of specific pre-mRNAs – RNA *trans*-splicing molecules (RTMs) – are the key players. These RTMs effect a *trans*-

splicing event between the target pre-mRNA and the RTM which is mediated by the spliceosome. An RTM carries three domains; i) a binding domain complementary to the target intron to localize the RTM to the target pre-mRNA; ii) a splicing domain containing splicing elements for efficient *trans*-splicing; and iii) a coding domain comprising one or more wildtype exons that are *trans*-spliced to the target. The *cis*-splicing elements and the binding domain are not retained in the modified RNA product [71]. Depending on the gene portion to replace, SMaRT can be divided into 3', 5' or internal exon replacement [69]. See Figure 6.

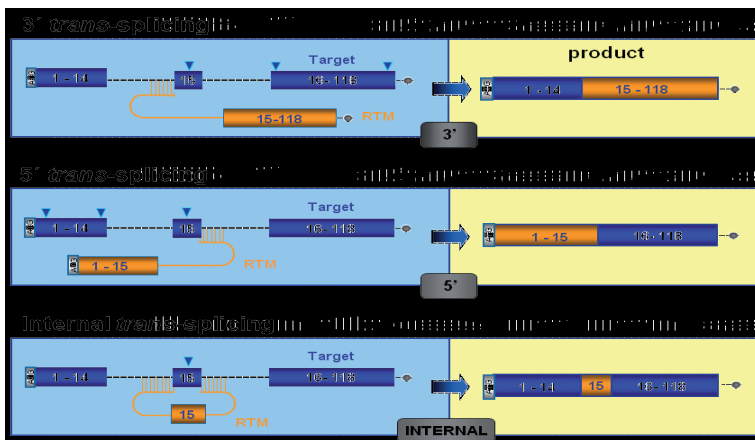


Figure 6. Schematic overview on different applications of SMaRT. **A: 3' *Trans*-splicing:** If there is a mutation in the 3' part of the target gene a wildtype mRNA can be obtained by 3' splicing. Therefore, a 3' RTM with a binding domain situated in the intron 5' to the first exon to be exchanged is necessary. E.g. if the mutation to be corrected is in exon 15, a binding domain for intron 14 is designed. This RTM can correct mutations more 3' as well. After binding of the RTM the two mRNAs are *trans*-spliced and combined into a wildtype mRNA. **B: 5' *Trans*-splicing:** If correction of a mutation in the 5' part of a gene is desired a 5' RTM with a binding domain located in the intron 3' to the exon to be exchanged is created. If the mutation to be corrected with 5' splicing is in exon 15, a RTM with a binding domain in intron 15 is required. This RTM can repair mutations more 5' than exon 15 as well. **C: Internal *Trans*-splicing:** There is also a method to exchange only one exon, called internal *trans*-splicing or internal exon replacement (IER). Here an RTM with two binding domains and 5' and 3' splice elements is applied. Arrowheads indicate mutations.

10.3. Efficiency of SMaRT

The efficiency of *trans*-splicing to correct genetic defects and acquired disorders at pre-mRNA level has already been demonstrated for 3' as well as for 5' *trans*-splicing in different diseases *in vitro* and *in vivo*. See Table 3.

Author	Approach	Year
3' trans-splicing		
Puttaraju et al.	3' repair of lacZ in a tractable system	2001
Chao et al.	3' repair of haemophilia A mice in vivo	2003
Dallinger et al.	3' repair in a lacZ model system in a keratinocyte specific background	2003
Liu et al.	3' repair of CFTR mRNA (adenovirally)	2005
Rodriguez-Martin et al.	3' reprogramming of tau alternative splicing in a model system	2005
Zayed et al.	3' repair of DNA-PKcs in SCID (delivery via sleeping beauty)	2007
Chen et al.	3' repair dystrophin myotonic type 1 pre-mRNA	2008
Coady et al.	3' SMN2 trans-splicing in combination with blocking an cis-splice site in mice in vivo	2010
Muraier et al.	Functional 3' repair of the COL7A1 gene	2010
Wang et al.	3' introduction of therapeutic proteins in highly abundant albumin transcripts in mice in vivo	2009
Gruber et al.	3' reprogramming of tumor marker genes to introduce suicide genes	2011
5' trans-splicing		
Mansfield et al.	5' repair of CFTR mRNA	2000
Kierlin-Duncan et al.	5' repair of β -globin mRNA	2007
Wally et al.	5' repair of the PLEC1 gene	2007
Wally et al.	5' K14 mRNA reprogramming	2010
Rindt et al.	5' trans-splicing repair of huntingtin at mRNA level	2012
Internal trans-splicing		
Lorain et al.	Exon exchange approach to repair Duchenne dystrophin transcripts in a minigene	2010
Koller et al.	A screening system for IER molecules	2011

Table 3. Overview on functional *trans*-splicing approaches so far.

RNA *trans*-splicing for gene correction is usually performed by 3' RNA *trans*-splicing to exchange 3' coding parts of a gene of interest. 3' RNA *trans*-splicing was successfully applied to restore wildtype gene expression pattern amongst others in patient cells or in animal models of epidermolysis bullosa, cystic fibrosis, X-linked immunodeficiency and hemophilia A [66,72,73]. Primarily co-transfection experiments with RTMs and artificial targets were used to give proof of principle of functionality of the *trans*-splicing process.

So a tractable lacZ model repair system, in which user defined target introns can be *trans*-spliced into a mutated lacZ gene to test target specific 3' RTMs by double transfection in 293T cells was developed by Puttaraju et al.. Functional lacZ correction was detected on mRNA and protein level by qRT-PCR and western blotting for one CFTR intron [74]. Chao et al. showed that the hemophilia A phenotype in factor VIII (FVIII) knockout mice can be repaired by the introduction of a 3' RTM. After delivery of the DNA through the portal vein, the FVIII protein was detected by western blot analysis of cryoprecipitated murine plasma. Long-term correction was shown via adenoviral tail vein transduction of the specific RTM. In the classical tail-clip test all naive knockout mice died, whereas eight out of ten treated mice survived, indicating that 3' *trans*-splicing is suitable to correct the bleeding disorder in hemophilia A [73]. Liu et al. used a recombinant adeno-associated viral vector system to target the human cystic fibrosis (CF) polarized airway epithelia from the apical membrane. The measurement of the cAMP-sensitive short circuit currents levels confirmed the CFTR correction by SMaRT [75]. Dalinger et al. showed as a proof of principle in the skin the correction of the EB-associated gene *COL17A1* by 3' *trans*-splicing. Using a lacZ model repair system, an intron specific target molecule and a rationally designed RTM, the feasibility of SMaRT was shown by co-transfection experiments in keratinocytes [76]. Using a minigene Rodriguez-Martin et al. published functional 3' *trans*-splicing on mRNA level after double transfection of the minigene and specific 3'RTM in COS-7 and SH-SY5Y cells for tau mRNA [77]. Zayed et al. demonstrated 3' correction of the DNA protein kinase catalytic subunit (DNA-PKcs) gene, which is responsible for severe combined immune deficiency (SCID). Specific 3' RTMs were transfected into scid.adh cells using the Sleeping Beauty transposon system. After this treatment irradiated cells showed an 4.3 fold increase of surviving cells over irradiated untreated scid.adh cells. Correction of the mutation was shown via QRT-PCR and sequencing on mRNA level. Additionally, functional 3'*trans*-splicing was detected on mRNA level via sequencing and on protein level via western blotting in SCID multipotent adult progenitor cells [78]. Chen et al. corrected the dystrophin myotonic protein kinase gene responsible for the most common muscular dystrophy in adults by 3' *trans*-splicing on mRNA level [79]. Coady et al. showed *in vivo* correction of spinal muscular atrophy (SMA) by 3'*trans*-splicing in mice recently. A single injection of a repair construct *trans*-splicing *SMN2* carried by a PMU3 vector into the intracerebral-ventricular space of SMA neonates lessens the severity of the SMA phenotype in a severe mouse model and extends survival by around 70% [80]. Murauer et al. corrected mutations in *COL7A1* by 3'*trans*-splicing. RDEB keratinocytes retrovirally transduced with a 3'*trans*-splicing molecule showed an increase of *COL7A1* mRNA sqRT-PCR and recovery of full-length type VII collagen expression on protein level in western blot and

immunofluorescent staining. Moreover normal morphology and reduced invasive capacity was achieved in transduced cells. Correct localization of type VII collagen at the basement membrane zone in skin equivalents, where it assembles into anchoring fibril like structures, showed the potential of *trans*-splicing to correct an RDEB phenotype *in vitro* [66]. There are also alternative approaches in which therapeutic proteins are produced after specific 3' *trans*-splicing events into highly abundant albumin transcripts using 3' RTMs [81]. Another area of application of SMaRT was performed by Gruber et al. to treat malignant SCC tumors, which are life-threatening issues for RDEB patients. The transfection of RDEB SCC cells with a designed 3' RTM lead to the fusion of the toxin streptolysin O, carried by a 3' RTM, to MMP-9 pre-mRNA molecules, resulting in the expression of the toxin and subsequently to the cell death of transfected tumor cells [82].

5' *trans*-splicing to correct upstream coding sequences of an mRNA of interest was first shown by a double transfection model to repair mutations in the cystic fibrosis transmembrane receptor (CFTR) pre-mRNA. Functionality tests were performed by anion efflux transport measurements. RTMs were designed capable to repair the 5' portion of CFTR transcripts [83]. 5' *trans*-splicing was also applied for the substitution of exon 1 of β -globin in cells co-transfected with a target molecule and an RTM in 293T cells and lead specific *trans*-splicing detected by one step RT-PCR [84]. Endogenous 5' *trans*-splicing induced gene correction was first demonstrated by Wally et al. on the basis of the *PLEC* gene involved in the disease epidermolysis bullosa simplex (EBS). Restoration of wild-type plectin expression patterns was shown by immunofluorescence microscopy of patient fibroblasts after RTM treatment [61]. Additionally, exons 1–7 of the keratin 14 gene (*KRT14*) were replaced in an autosomal dominant model of EBS resulting in recovery of K14 on RNA and protein level, detected by SQRT-PCR, western blotting and immunofluorescence staining by transient transfection of specific 5' RTMs chosen in a screening procedure [85]. Recently 5' *trans*-splicing correction of a disease causing huntingtin allele on mRNA level was reported by Rindt et al. [86].

Lorain et al. primarily published the methodology of internal exon replacement (IER) to correct a dystrophin minigene on mRNA level [87]. Recently, Koller et al. developed a new RTM screening system to improve double RNA *trans*-splicing for the correction of the EB associated gene *COL17A1* [88].

10.4. RTM screening systems

So far, there are no general rules for the design of highly efficient *trans*-splicing RTMs. However, recent studies revealed the influence of minor differences in length, composition and localization of the binding domain (BD) on RTM efficiency and specificity [85,88]. Due to the fact that an RTM can't be predicted rationally, we established a fluorescence-based screening system to select an efficient RTM from a pool of randomly designed RTMs. This screening system is composed of fluorescence-based RTM backbones, in which randomly created binding domains are cloned, and a gene specific target molecule. The target binding region (exon/intron sequence of a gene of interest) is PCR amplified, randomly fragmented and cloned into the RTM vector. The coding region consists

of a fluorescence reporter, divided into two (5' or 3' *trans*-splicing) or three parts (internal exon replacement) and distributed to both screening molecules (target molecule and RTM). The RTM library is composed of individual RTMs with various binding domains. Their efficiency can be evaluated by fluorescence microscopy and flow cytometry. For flow cytometric analysis, individual selected RTMs of the RTM library are co-transfected with the designed target molecule, including the full-length target binding region, and the missing sequence of the split fluorescence reporter into HEK293FT cells. Co-transfection of RTM and target molecule into HEK293FT cells results in the restoration of expression of the fluorescence reporter. The intensity of the fluorescence signal of the reporter molecule gives information on the functionality of the binding domain. The most efficient BDs can be tested for endogenous experiments in patient cells. After transfection of the screening-RTM, the fusion of the splitted *trans*-splicing reporter and the endogenous target is detected by RT-PCR. To develop an mRNA based gene therapy an RTM, carrying the wildtype sequence instead of the coding sequence of the fluorescence molecule, is constructed. After RTM treatment of patient cells a mutated gene part is exchanged by *trans*-splicing and wildtype transcripts are restored.

11. RTMs for the murine COL7A1 gene

We started to establish 5' *trans*-splicing for murine *COL7A1* in order to analyze the functionality of RNA *trans*-splicing *in vivo*, due to the existence of a mouse model carrying a neo cassette in intron 2 of *COL7A1* generating aberrant splice variants, which lead to a reduction of type VII collagen expression [46]. By close similarity of this mouse model to the human RDEB phenotype and location of the defect in the 5' part of *COL7A1*, this mouse model exhibits obviously an ideal system to test our 5' repair molecules and investigate different application strategies.

Intron 15 was chosen as target intron because its size of about 1,5kb allows to create a large number of different binding domains. To generate a large amount of different RTMs, containing binding domains with different binding affinities to the target intron, the target exon/intron was digested out of the artificial target used in the screen with HindIII and BamHI and digested with CviJI*. The resulting fragments with a length of 50-750bp were cloned into the RTM backbone. Binding domains were identified by colony PCR using a forward primer situated in the 5' half of the split GFP and a vector specific reverse primer. Possible binding domains with different lengths were detected on a 2% agarose gel after gel electrophoresis. To check orientation and location of the binding domain, clones with inserts were sequenced. To evaluate the created RTM library the artificial target containing the target intron (intron15) and the 3' half of the split AcGFP instead of the 3' part of murine *COL7A1* was cotransfected with the RTM library respectively individual RTMs into HEK293FT. The RTMs contain a transfection reporter (DsRED), the 5' half of the split AcGFP instead of the first 15 exons of *COL7A1* and variable binding domains. The cotransfected cells were analyzed concerning their AcGFP/DsRED expression ratios by fluorescence

microscopy and flow cytometry, whereby a higher ratio indicates the presence of a more functional binding domain in the RTM. See Figures 7+8.

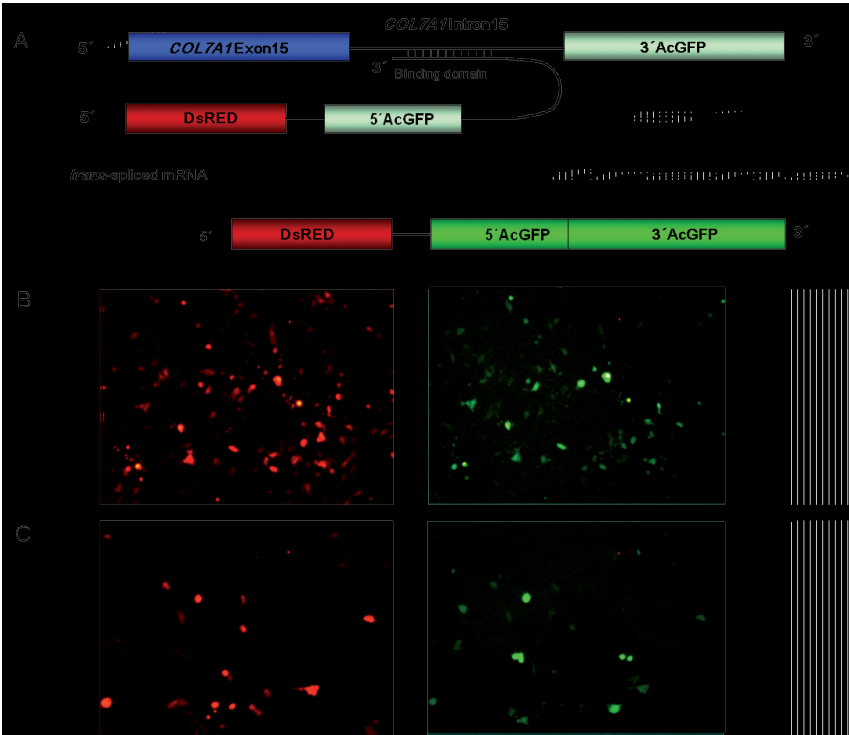


Figure 7. Fluorescence microscopy of with RTM library and target double transfected HEK293 cells. **A:** Functional binding domains lead to specific *trans*-splicing of the two pre mRNAs which are then combined into one mRNA containing DsRED and full-length AcGFP. Red fluorecence indicates the transfection of a RTM in the cells whereas red and green fluorecence indicates functional *trans*-splicing. **B:** Double transfection of an artificial target containing exon/intron 15 of murine COL7A1 and an RTM library for this exon/intron in HEK293FT. **C:** Double transfection of an artificial target containing exon/intron 15 of murine COL7A1 and the best RTM for this intron.

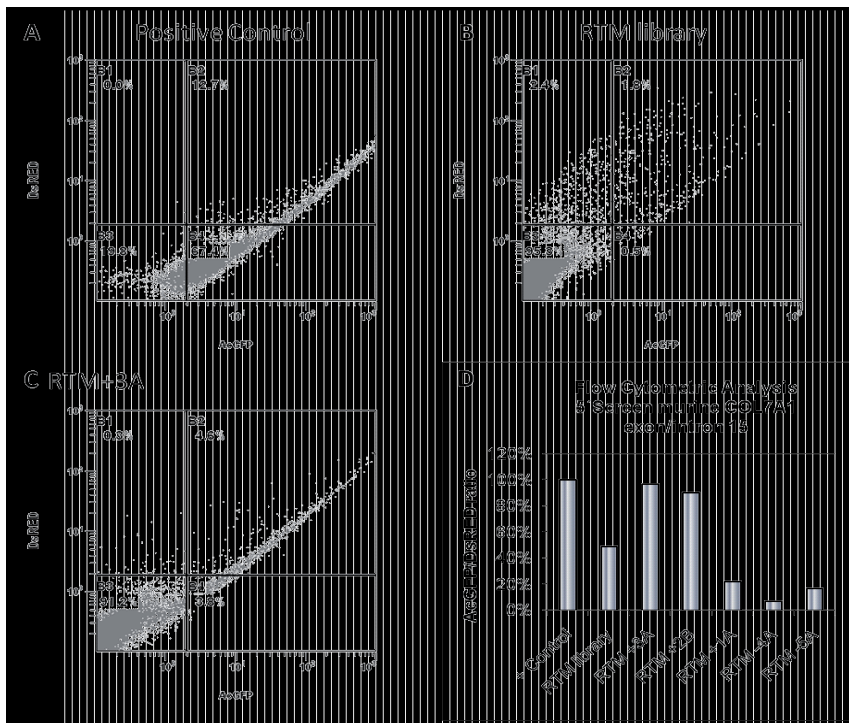


Figure 8. Flow cytometric analysis: 5' screen for murine *COL7A1* exon/intron 15. Red fluorescence (shown on Y-axis) indicates transfection of RTMs in the cells; green fluorescence (shown on X-axis) indicates specific *trans*-splicing. **A:** The positive control is a vector containing a DsRED linker AcGFP construct. This FACS plot mimics the AcGFP and DsRED ratios expected from the product of optimal *trans*-splicing. **B:** The RTM library shows a *trans*-splicing efficiency (AcGFP/DsRED ratio) of 48,94% calculated from *trans*-splicing positive cells/all transfected cells. (B2 1,8% + B4 0,5%)/(B1 2,4% + B2 1,8% + B4 0,5%) The fact, that several cells seem to be exclusively green can be explained by the intense AcGFP fluorescence, which tends to override the weaker DsRED fluorescence. **C:** The most efficient RTM analyzed, with a *trans*-splicing efficiency of 96,55%, (B2 4,6% + B4 3,8%)/(B1 0,3% + B2 4,6% + B4 3,8%) shows a dot plot pattern similar to the positive control. Therefore the binding domain of RTM +3A was chosen to be used in further endogenous experiments. **D:** A Comparison of AcGFP/DsRED ratios of single RTMs containing different binding domains, shows a wide variability of AcGFP/DsRED ration spanning less than 10% to nearly 100%.

The RTM with the highest AcGFP/DsRED ratio (RTM+3A) was chosen for further endogenous experiments. To check endogenous functionality of the RTM was transiently transfected into an immortalized murine keratinocyte cell line [46]. The 5' part of the split AcGFP contained by the screening RTM was specifically *trans*-spliced with its endogenous target – the murine *COL7A1* mRNA – resulting in a AcGFP-*COL7A1* fusion mRNA detected by RT-PCR analysis and subsequent sequencing. See Figure 9.

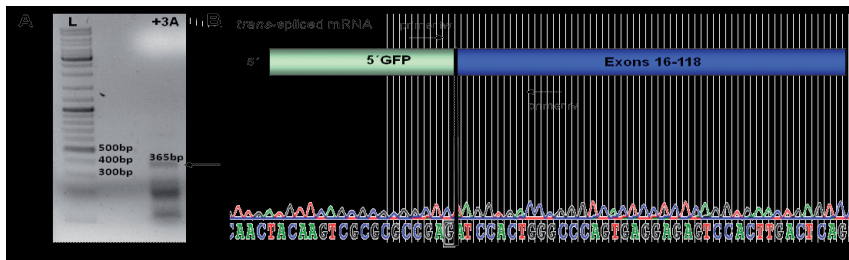


Figure 9. Endogenous *trans*-splicing into exon/intron 15 of murine *COL7A1*. **A:** RT-PCR analysis of transiently RTM+3A transfected spontaneously immortalized mouse wildtype keratinocytes [46] using primers in the 5' part of split AcGFP and in exon 18 of *COL7A1* resulted in detection of a 365bp band after agarose gel-electrophoresis. **B:** The fragment was verified to be an AcGFP-*COL7A1* fusion by sequencing. **+3A** cDNA analysis of spontaneously immortalized mouse wildtype keratinocytes transiently transfected with RTM +3A **L** Ladder Mix DNA marker

12. Conclusion

RNA *trans*-splicing is a useful methodology to reprogram genes for diagnostic and therapeutic purposes. Due to a variety of advantages over traditional gene-replacement strategies, RNA *trans*-splicing is used to correct the phenotype of many genetic diseases *in vitro*, ranging from epidermolysis bullosa to neurodegenerative diseases. *In vivo* studies are in progress to accelerate the way to the medical use of this RNA-based application.

We have established all three modes of *trans*-splicing (5', 3' and internal exon replacement) in our laboratory on the basis of several EB-associated genes (*KRT14*, *PLEC*, *COL7A1*, *COL17A1*). In this work we focused on the methodology of 5' RNA *trans*-splicing to correct mutations localized within the first 15 exons of the murine *COL7A1* gene encoding for type VII collagen. *COL7A1* is a large gene with over 9kb and is therefore suitable for this approach, in which only a short RTM has to be designed, harbouring only the first 15 exons of the gene. Using an RTM screening system, described by Wally et al 2011 [89], it should be possible to increase the *trans*-splicing efficiency of designed RTMs to a level where the phenotype of *COL7A1* deficient cells can be converted into wildtype. We analyzed the binding properties of randomly designed RTMs specific for intron 15 of murine *COL7A1* and tested the most efficient RTM in *COL7A1* deficient keratinocytes for endogenous functionality. The RTM was able to induce endogenous 5' *trans*-splicing into murine *COL7A1* pre-mRNA molecules, manifested in the fusion of the 5' GFP part of the RTM with exon 16 of *COL7A1*. Next steps are the exchange of the 5' GFP part by the 5' sequence of murine *COL7A1* (exons1-15) and to investigate if our RTMs are able to increase the level of full-length *COL7A1* mRNA leading to the recovery of functional type VII collagen in *COL7A1* deficient cells and in skin equivalents. In summary we demonstrated a novel RNA-based strategy to correct disease-associated mutations within *COL7A1*, thereby avoiding or minimizing many problems present in standard cDNA gene therapies including fragmentation of the large *COL7A1* gene, the size limitation of the transgene and over- and ectopic expression of the transgene.

The development of a gene therapy for type VII collagen deficiency would increase the chance to find a cure for dystrophic EB. Additionally, the improvement of the methodology of 5' RNA *trans*-splicing will help us to move closer to the treatment of other genetic diseases caused by mutations in especially large genes.

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Molecular Therapy for Lysosomal Storage Diseases

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Additional information is available at the end of the chapter

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1. Introduction

Lysosomes are organelles involving the catabolism of biomolecules extracellularly and intracellularly incorporated, which contain more than 60 distinct acidic hydrolases (lysosomal enzymes) and their co-factors. Lysosomal storage diseases (LSDs) are caused by germ-line gene mutations encoding lysosomal enzymes, their activator proteins, integral membrane proteins, cholesterol transporters and proteins concerning intracellular trafficking of lysosomal enzymes [1,2]. The LSDs associate with excessive accumulation of natural substrates, including glycoconjugates (glycosphingolipids, oligosaccharides derived from glycoproteins, and glycosaminoglycans from proteoglycans) as well as heterogeneous manifestations in both visceral and nervous systems [1,2]. LSDs comprise greater than 40 diseases, of which incidence is about 1 per 100 thousand births, and recognized as so-called 'Orphan diseases'.

In the biosynthesis of lysosomal matrix enzymes, newly synthesized enzymes are N-glycosylated in the endoplasmic reticulum (ER) and then phosphorylated in the Golgi apparatus on the 6 position of the terminal mannose residues (M6P) via two step reactions catalyzed by Golgi-localized phosphotransferase and uncovering enzyme necessary to expose the terminal M6P residues [3,4]. The M6P-carrying enzymes then bind the cation-dependent mannose 6-phosphate receptor (CD-M6PR) at physiological pH in the Golgi. The enzyme-receptor complex is then transported to late-endosomes where the M6P-carrying enzymes dissociate from the receptor at acidic pH, while the CD-M6PR then traffics back to the Golgi as a shuttle. M6P-carrying enzymes are delivered to lysosomes via fusion with late-endosomes. A small percentage of lysosomal enzymes is known secreted from the cell. The secreted M6P-carrying enzymes or the dephosphorylated enzyme with terminal mannose residues can then bind either cation-independent M6P/IGFII receptor (CI-M6PR) or mannose receptor (MR) on the plasma membrane [4,5]. Thus, the extracellular lysosomal enzymes can be endocytosed via both glycan receptors to be delivered to the lysosomes where the captured enzymes can exhibit their normal catabolic functions.

Many therapeutic approaches developed for LSDs, including bone marrow transplantation (BMT), stem cell-based therapy (SCT), enzyme replacement therapy (ERT) and *ex vivo* gene therapy, are based on this physiologic secretion/uptake system (cross-correction). In successful intravenous ERT for LSDs involving mainly visceral symptoms, including type 1 Gaucher disease [6,7] and mucopolysaccharidosis I (MPS I) [8], MPS VI [9], Fabry [10,11], and Pompe diseases [12,13], either MR or CI-M6PR have been utilized as delivery targets of the recombinant lysosomal enzyme drugs produced by mammalian cell lines including CHO cells and human fibrosarcoma cells. However, intravenous ERT has several disadvantages: i) long-life therapy, ii) requirement of large amounts of recombinant human enzymes, iii) high cost, iv) immune response to the exogenous enzymes [14], and v) ineffectiveness towards LSDs involving neurological signs because of the blood-brain barrier (BBB), although clinical trials are under-going of intrathecal ERT for MPS type I [15], II and IIIB patients. SCT using hematopoietic stem cell (HSC), hematopoietic precursor cell (HPC) and mesenchymal stem cell (MSC) derived from bone marrows has also been utilized as a treatment for LSDs animal models and patients [16-20]. BMT and SCT are based on that stem cells distribute widely *in vivo* as sources continuously producing the deficient enzymes. However, application of BMT is generally limited to LSDs that show a clear beneficial response and for which ERT is not available.

On the other hand, the gene replacement therapy (GT) [21-24] has advantages, including i) long-lasting therapy by a single transduction utilizing recombinant viral gene transfer vectors [25-29], ii) cross-correction effects, and iii) possible CNS-directed application to LSDs involving neurological symptoms [23,24,30-33], whereas GT has disadvantages, including i) low levels and persistence of expression in all tissues of patients, ii) incomplete response to therapy dependent on clinical phenotypes, and iii) insertional mutagenesis resulting in neoplasia. GT is one of the promising therapeutic approaches, especially toward LSDs involving CNS symptoms. In this review, we focus on the challenges to develop the CNS-directed GT for LSDs including GM2 gangliosidoses.

2. GM2 gangliosidoses

Lysosomal β -hexosaminidase (Hex, EC 3.2.1.52) is a glycosidase that catalyzes the hydrolysis of terminal N-acetylhexosamine residues at the non-reducing ends of oligosaccharides of glycoconjugates [34,35]. There are two major Hex isozymes in mammals including man, HexA ($\alpha\beta$, a heterodimer of α - and β -subunits) and HexB ($\beta\beta$, a homodimer of β -subunit), and a minor unstable isozyme, HexS ($\alpha\alpha$, a homodimer of α -subunit). All these Hex isozymes can degrade terminal β -1,4 linked N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) residues, while only HexA and HexS prefer negatively charged substrates and cleave off the terminal N-acetylglucosamine 6-sulfate residues in keratan sulfate. Hex A is essential for cleavage of the GalNAc residue from GM2 ganglioside (GM2) in co-operation with GM2 activator protein (GM2A) [34,35].

Tay-Sachs disease (TSD) (MIM 272800) and Sandhoff disease (SD) (MIM 268800) are autosomal recessive GM2 gangliosidoses caused by germ-line mutations of *HEXA* (locus 15q23-24)

encoding the Hex α -subunit, and *HEXB* (locus 5q13) encoding the Hex β -subunit, respectively [34,35]. The genes exhibit sequence homology, and the gene products exhibit 57% similarity in amino acid sequence. In TSD, the genetic defect of *HEXA* causes a deficiency of HexA ($\alpha\beta$ with excessive accumulation of GM2 in the central nervous system (CNS), resulting in neurological disorders, including weakness, startle reaction, early blindness, progressive cerebellar ataxia, psychomotor retardation, and cherry red spots, and macrocephaly. In SD, the inherited defect of *HEXB* leads to simultaneous deficiencies of HexA and HexB with accumulation of GM2 in the CNS and of oligosaccharides carrying the terminal N-acetylhexosaminyl residues at their non-reducing ends, resulting in involvement of the visceral organs including cardiomegaly and minimal hepatosplenomegaly as well as the neurological symptoms. GM2 gangliosidosis AB variant (MIM 272750) is very rare autosomal recessive LSD caused by the gene mutation of GM2 activator protein (*GM2A* locus 5q31.3-q33.1) [34,36]. The gene product GM2A specifically binds GM2 to pull up from membranes in lysosomes, and present it to HexA for degradation of GM2. The deficiency of GM2A also cause the GM2 accumulation and neurological symptoms similar to those of TSD and SD [34,36]. The pathogenic mechanisms of these GM2 gangliosidoses has not been fully elucidated, although neurodegeneration and neuroinflammation have been reported to contribute to the pathogenesis [34,35,37,38].

GM2 gangliosidoses including TSD and SD exhibit a spectrum of clinical phenotypes, which vary from the severe infantile form (classical type), which is of early onset and fatal culminating in death before the age of 4 years, to the late-onset and less severe form (atypical type), which allows survival into childhood (subacute form) or adulthood (chronic form) [34,35,37,38]. Many mutations have been identified for each gene, including missense, deletion and insertion mutations [34,39-41]. Structural information on the basis of the crystal structures of human Hex B [42,43] and HexA [44] allowed us to predict the effects of missense mutations identified in TSD [34,39,40] and SD [34,39,41] on the protein structures of mutated gene products. According to these reports, the β -subunit of Hex comprises two domains (domain I and II). Domain I has an α/β topology, and domain II is folded into a $(\beta/\alpha)_8$ -barrel with the active site pocket at the C-termini of the β -strands. An extrahelix that follows the eighth helix of the $(\beta/\alpha)_8$ -barrel is located between domain I and the barrel structure. Only the α -subunit active site can hydrolyze GM2 due to a flexible loop structure that is removed post-translationally from β , and to the presence of α N423 and α R424. The loop structure is involved in binding the GM2A, while α R424 is critical for binding the carboxylate group of the N-acetylneuraminic acid residue of GM2. The β -subunit lacks these key residues and has β D452 and β L453 in their place. The β -subunit therefore cleaves only neutral substrates efficiently. The representative amino acid substitutions have been reported in the α -subunit, including R170W, R178H, W420C, C458Y, L484P, R499C/H, and R504C/H, as well as in the β -subunit, R505Q and C534Y. The dysfunctional and destabilizing defects in Hex α - and β -subunits well reflect biochemical and phenotypic abnormalities in TSD and SD, respectively. Such structural information should be useful to develop novel therapeutic approaches for these disorders [34,45].

3. General aspects of gene therapy for LSDs

Gene therapy (GT) utilizing various vectors for gene transfer has been preclinically and clinically applied for LSDs in recent years [21-33]. Recombinant viral vectors including retroviruses [25,32], adenovirus [26-28], herpes simplex virus (HSV) [33], adeno-associated virus (AAV) [29,46-48] and lentiviruses [24,49-51] are utilized currently as the effective means of gene transfer and enzyme expression. The retroviruses have been used primarily in *ex vivo* applications to transduce the dividing cells, such as HPC, HSC and other stem cells in culture, which are then transplanted into a recipient. However, the retroviral vectors are not suitable for *in vivo* GT due to lack of ability to transduce non-dividing cells. On the other hand, the adenoviruses can infect very efficiently non-dividing cells. However, the use of the early generation adenoviral vectors has been limited due to their strong antigenicity. In contrast, lentiviruses can infect both dividing and non-dividing cells, and are applicable to both *ex vivo* and *in vivo* GT. AAV vectors are able to transduce many cell types *in vivo* effectively, and it is often used as a safer tool for gene transfer because of the lower immunogenicity.

The application of recombinant viral vectors varies dependently on several factors, including ease of vector delivery, expression level in cell types and target tissues and organs mainly affected with LSD. At initial stage of development of GT for LSDs, the *ex vivo* transduction of HPC derived from type 1 Gaucher disease [25] and fibroblasts obtained from MPS VII model mice [32] using retroviral vectors was successful to secrete high levels of the enzymes and corrected the deficiencies. The *ex vivo* GT using retroviral vector and autologous HSC or HPC (human CD34+ cells) derived from bone marrow of the patient as donor cells for transplantation was clinically applied to type 1 Gaucher disease patients, and demonstrated the production of therapeutically effective levels of enzyme activity, resulting in persistent circulating enzyme available to tissues and organs [52]. The transduced cells also migrated into many tissues, expressed high levels of enzyme and reduced lysosomal storage in several critical tissues. However, several problems had been emerged, including less efficiency in transduction of human HSC or HPC using murine-based retroviral vector and difficulty in continuous production of sufficient amounts of recombinant enzymes to maintain the effectiveness.

The lentiviral vector based on human immunodeficiency virus had been expected to overcome the limitation of early generation of murine-based retroviral vectors in *ex vivo* GT [53]. Transduction efficiency of human HPC derived from Gaucher disease patient [54] was improved by using HIV-based lentiviral vector. β -Glucuronidase (GUSB)-deficient mobilized peripheral blood CD34(+) cells from a patient with MPSVII were transduced with a third-generation lentiviral vector encoding human GUSB, and then xenotransplantation to murine model with MPSVII. The corrected cells distributed widely throughout recipient tissues, resulting in significant therapeutic effects including improvements in biochemical parameters and reduction of the lysosomal distension of several host tissues [24].

Direct *in vivo* GT using adenoviral vector have been preclinically applied to murine models with Pompe, Fabry, and Wolman diseases, resulted in sufficient expression of circu-

lating enzymes and reduction of storage materials in the affected tissues [27,55, 56]. However, these therapeutic effects were transient because of the severe immune reactions directed against the adenoviral vector. Single intravenous administration of a modified adenoviral vector to Pompe disease mice was demonstrated to reduce glycogen storage with minimal immune response [57].

The AAV vector has been also developed as an alternative gene transfer tool for direct *in vivo* GT for LSDs. Intramuscular injection of AAV2 serotype vector [58,] in the murine models of Pompe disease, Fabry disease and MPS VII caused high level expression in the muscle tissues but lower levels of circulating enzyme activity [59-61], although the efficacy varied depending on the diseases. Intravenous injection of AAV2 vectors in young adult mice with MPS VII and Fabry disease reduced the lysosomal storage in many tissues [61,62] Significant improvement was observed in MPS VII and MPS I mice following intravenous delivery of AAV2 vector during the neonatal period [28,63]. These findings suggested the effectiveness of AAV vector delivery at early presymptomatic stage to prevent onset rather than delayed intervention for progressive LSDs.

As mentioned above, GT has therapeutic potency for LSDs involving neurological symptoms superior to that of clinically applied intravenous ERT, in which the enzyme cannot cross the BBB. Several CNS-directed *ex vivo* and *in vivo* GT have been performed for animal models of LSDs with brain involvement. Genetically modified bone marrow stromal cells using retroviral vector improved CNS pathology and cognitive function in MPS VII and GM1-gangliosidosis mice following intraventricular transplantation [64, 65]. Genetically modified human neuronal precursor cells (NPCs) differentiated into neurons and astrocytes and expressed β -glucuronidase for at least 6 months after injection into striata of adult MPS VII model mice. However, the cells did not migrate and correction was limited to regions adjacent to the transplantation site [66] *In vivo* GT of metachromatic leukodystrophy (MLD) by lentiviral vector corrected neuropathology and protected against learning impairments in the model mice [49]. CNS-directed *in vivo* GT using AAV vectors have been demonstrated to have therapeutic effects on the mouse model of LSDs involving neurological signs, including MPS IIIB [67], MPS VII [68], Globoid cell leukodystrophy (GLD) [69], Nieman-Pick A (NPA)[70] and α -mannosidosis [71] by intracranial administration of recombinant AAV vectors. Thus, AAV vectors exhibit a number of properties that have made this vector system an excellent choice for both CNS gene therapy and basic neurobiological investigations. *In vivo*, the preponderance of AAV vector transduction occurs in neurons where it is possible to obtain long-term, stable gene expression with very little accompanying toxicity. Promoter selection, however, significantly influences the pattern and longevity of neuronal transduction distinct from the tropism inherent to AAV vectors. AAV vectors have successfully manipulated CNS function using a wide variety of approaches including expression of foreign genes, expression of endogenous genes, expression of antisense RNA and expression of RNAi. With the discovery and characterization of different AAV serotypes, as well as the creation of novel chimeric serotypes, the potential patterns of *in vivo* vector transduction have been expanded substantially, offering alternatives to the more studied AAV 2 serotype. Furthermore, the development of specific AAV chimeras offers the potential to further re-

fine targeting strategies. These different AAV serotypes also provide a solution to the immune silencing that proves to be a realistic likelihood given broad exposure of the human population to the AAV 2 serotype. These advantageous CNS properties of AAV vectors have fostered a wide range of clinically relevant applications including Parkinson's disease, lysosomal storage diseases, Canavan's disease, epilepsy, Huntington's disease and ALS. In many cases the proposed therapies have progressed to phase I/II clinical trials. Each individual application, however, presents a unique set of challenges that must be solved in order to attain clinically effective gene therapies [72].

4. Gene therapy for GM2 gangliosidoses

4.1. Experimental and preclinical gene therapy using animal models

GM2 gangliosidoses, including Tay-Sachs disease (TSD), Sandhoff disease (SD) and the AB variant disorder, are characterized by excessive accumulation of GM2 and neurological symptoms due to progressive neurodegeneration and gliosis, as described above. However, there is no effective therapy for GM2 gangliosidoses at present, although we have reported and proposed the clinical potential of the intrathecal ERT using recombinant modified human HexA [73] and HexB [74,75] in recent years. It is crucial for treatment of GM2 gangliosidoses to develop the CNS-directed molecular therapy including such intrathecal ERT, *ex vivo* and *in vivo* GT or the combined methods including SRT [76]. In this chapter, we would focus on the CNS-directed GT and summarize the preclinical approaches using small and large animal models with GM2 gangliosidoses.

At early stage of development of GT for GM2 gangliosidoses, gene transduction of cultured cells was performed by utilizing recombinant vectors (virus or plasmids), and examined the effect of cross correction due to the secreted Hex isozymes. Guidotti, JE. *et al.* constructed a retroviral vector encoding for the α -subunit of human HexA (HEXA cDNA) and transduced the HexA-deficient fibroblasts derived from Tay-Sach disease model mice (*Hexa*^{-/-} mice) [77]. Transduced cells overexpressed the human Hex α -subunit to produce the chimeric HexA composed of human α -subunit and murine β -subunit, which were taken up via CI-M6PR by non-transduced cells and exhibited the cross-correction effect.

On the other hand, Martino *et al.* also constructed a retroviral vector encoding for the α -subunit of human HexA (HEXA cDNA) and transduced NIH3T3 murine fibroblasts, resulting in production of large amount of human Hex activity. The secreted Hex was incorporated into the fibroblasts derived from TSD patient, but failed to correct intracellular GM2 storage, probably because of the absence of HexA isozyme sufficient for degrading the accumulated GM2 [78]. Akli S *et al.* produced a replication-deficient recombinant adenovirus (AdRSV) coding the human HEXA cDNA, and transduced the fibroblasts derived from TSD patients. Transduced cells restored the Hex activity ranging from 40 to 84% of the normal, and secreted the Hex α -subunit, which were delivered to lysosomes and degraded the GM2 accumulated in TSD fibroblasts [79].

We transfected an expression vector plasmid coding the human *HEXB* cDNA to fibroblasts derived from Sandhoff disease mice (*Hexb*^{-/-} mice) and established a transformed murine cell line stably producing the human Hex β -subunit [80]. However, the GM2 accumulated in the transformed murine cell line was not reduced, while co-transfection of the human *HEXA* cDNA resulted in restoration of HexA activity and reduction of GM2 storage.

Yamaguchi *et al.* evaluated the systemic *in vivo* GT for *Hexb*^{-/-} mice using cationic liposome-mediated plasmid using the *Hexb*^{-/-} mice [81]. The mice received a single intravenous injection of two plasmids, encoding the human α and β subunits of hexosaminidase cDNAs. As a result, 10–35% of normal levels of Hex expression, theoretically therapeutic levels, were achieved in most visceral organs, but not in the brain, 3 days after injection with decreased levels by day 7. Histochemical staining confirmed widespread enzyme activity in visceral organs. Both GA2 and GM2 were reduced by almost 10% and 50%, respectively, on day 3, and by 60% and 70% on day 7 compared with untreated age-matched *Hexb*^{-/-} mice.

These findings suggested that brain-directed *in vivo* GT based on direct transduction of the affected tissues by single gene transfer or *ex vivo* GT utilizing double genes (i.e. *HEXA* and *HEXB* cDNAs) for producing the homo-specific HexA should be required to achieve the therapeutic effects on TSD and SD. Since then, studies on the CNS-directed *in vivo* GT and *ex vivo* GT have been performed as two streams of development of molecular therapy for GM2 gangliosidoses.

4.2. CNS-directed *in vivo* gene therapy

Bourgoin *et al.* constructed the recombinant adenovirus coding the human *HEXB* cDNA, and transduced the fibroblasts derived from patient with SD resulting in high expression of HexA and HexB activities. They also administered the adenoviral vector intracerebrally to SD mice (*Hexb*^{-/-} mice), and succeeded in expression of near-normal level of enzymatic activity in the entire brain. Co-injection of hyperosmotic concentrations of mannitol with low doses of the adenoviral vector enhanced the vector diffusion in the injected hemisphere without viral cytotoxicity. It was suggested that such combination will allow a high and diffuse transduction efficiency of adeno-viral vector in the brain with higher safety [82].

Martino *et al.* also constructed a non-replicating herpes simplex viral (HSV) vector encoding *Hexa* cDNA. They transplanted the encapsulated recombinant HSV into the brain of *Hexa*^{-/-} mice. The diffusion of recombinant HSV and the secreted HexA derived from transduced neural cells corrected the GM2 storage in the brain during one month due to cross correction effects without adverse effects due to the viral vector [83].

Caillaud and co-workers reported that mono and bicistronic lentiviral vectors based on a simian immunodeficiency virus (SIV) containing the human *HEXA* or/and *HEXB* cDNAs were constructed and tested on the fibroblasts derived from the SD patient [84]. The bicistronic SIV.ASB vector encoding both *HEXA* and *HEXB* cDNAs enabled a massive restoration of Hex activity. A large reduction of GM2 accumulation in SIV.ASB transduced cells. Moreover, the Hex isozymes secreted by transduced SD fibroblasts were endocytosed in deficient cells via CI-M6PR, allowing GM2 metabolism restoration in cross-corrected cells.

Therefore, the bicistronic lentivector supplying both HexA and HexB isozymes may provide a potential therapeutic tool for the treatment of TSD and SD. A mechanistic link was demonstrated among GM2 accumulation, neuronal cell death, reduction of sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) activity, and axonal outgrowth. Arfi *et al.* examined the ability of the SIV.ASB vector to reverse these pathophysiological events, hippocampal neurons derived from embryonic *Hexb*^{-/-} mice, which were transduced with the lentival vector [85]. Normal axonal growth rates, the rate of Ca^{2+} uptake via the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) activity and the sensitivity of the neurons to thapsigargin-induced cell death were restored concomitantly with a decrease in GM2 and GA2 levels. Thus, the bicistronic SIV.ASB vector was revealed to reverse the biochemical defects and down-stream consequences in SD neurons, suggesting its potential of systemic and CNS-directed *in vivo* GT. Kyrkanides S. *et al.* performed the system *in vivo* GT utilizing the recombinant lentiviral vector FIV coding human *HEXB* cDNA to the neonatal *Hexb*^{-/-} mice via intraperitoneal administration. They also demonstrated the distribution of Hex isozymes into the CNS, including periventricular areas of the cerebrum as well as in the cerebellar cortex, and reduction of GM2 accumulated in these areas [86].

Cachon-Gonzalez *et al.* has reported that the *Hexb*^{-/-} mice treated by stereotaxic intracranial inoculation of recombinant adeno-associated viral (rAAV) vectors encoding the human *HEXA* and *HEXB* cDNAs, including an HIV tat sequence as a protein transduction domain (PTD), to enhance protein expression and distribution [87]. *Hexb*^{-/-} mice survived for >1 year with sustained, widespread and abundant enzyme delivery in the CNS. Onset of the disease was delayed with preservation of motor function; inflammation and GM2 storage in the brain and spinal cord was reduced. Gene delivery of the human HexA ($\alpha\beta$) by using AAV vectors has realistic potential for treating the TS and SD patients. Sargeant TJ. *et al.* demonstrated that intracranial co-injection of rAAV serotype 2/1 (rAAV2/1) vectors encoding the human *HEXA* and *HEXB* cDNAs prevents neuronal loss in the *Hexb*^{-/-} mice brain tissues, including thalamus, brainstem and spinal cord, and correlated with increased lifespan [88]. Moreover, they performed intracranial co-injection of rAAV2/1 vectors into 1-month-old *Hexb*^{-/-} mice [89]. As a result, the treated mice gave unprecedented survival to 2 years and prevented disease throughout the brain and spinal cord. Classical manifestations of disease, including spasticity were resolved by localized gene transfer to the striatum or cerebellum, respectively. Abundant biosynthesis of Hex isozymes and their global distribution via axonal, perivascular, and cerebrospinal fluid (CSF) spaces, as well as diffusion, account for the sustained phenotypic rescue—long-term protein expression by transduced brain parenchyma, choroid plexus epithelium, and dorsal root ganglia neurons supplies the corrective enzyme. Prolonged survival permitted expression of cryptic disease in organs not accessed by intracranial vector delivery.

4.3. CNS-directed *ex vivo* gene therapy

Ex vivo GT for GM2 gangliosidosis is based on the results of BMT previously reported [90,91]. BMT was demonstrated to prolong life span and ameliorate neurological symptoms in *Hexb*^{-/-} mice [90], and the synergistic effects was also shown in combination with substrate

reduction therapy (SRT) utilizing deoxynojirimycin derivatives [91]. Transduction of neural cells derived from *Hexa*^{-/-} and *Hexb*^{-/-} mice by recombinant viral vectors was performed.

Lacorazza HD *et al.* constructed the ecotropic retrovirus encoding the human *HEXA* cDNA and transduced multipotent neural progenitor cell lines, which stably expressed and secreted high levels of active HexA and cross-corrected the metabolic defect including GM2 storage in TSD fibroblastic cell line. The genetically engineered CNS progenitors were transplanted into the brains of both normal fetal and neonatal mice, in which substantial amounts of human Hex α -subunit and activity were observed throughout the brain enough for therapeutic effect in TSD [92].

Tsuji D. *et al.* constructed a recombinant lentiviral vector encoding the murine *Hexb* cDNA, and transduced microglial cells established from the brains of *Hexb*^{-/-} mice [50]. Transduced microglial cells produced and secreted Hex activity, in which the intracellularly accumulated GM2 and oligosaccharides with terminal N-acetylglucosamine residues were reduced. Transduced microglial cell line was expected as a donor for brain-directed *ex vivo* GT.

Mesenchymal stem cells (MSCs) derived from bone marrow stromal cells are one of the candidates for autologous donor cells for *ex vivo* GT, and have the multipotency to differentiate under specific culture conditions into other cell types such as osteoblasts, adipocytes, and chondrocytes [93], as well as into neural lineages [94]. Recently, we established MSCs derived from bone marrow of adult *Hexb*^{-/-} mice. The MSCs expressed cell-type specific markers, including CD29, CD90 and CD54, but not CD45, and exhibited the ability to differentiate into various cell types, including neuron-restricted precursor cells (NRPs) expressing NCAM carrying polysialic acid (PSA-NCAM). We produced a bicistronic retroviral vector (MSV-*modB*) encoding for the modified human *HEXB* cDNAs (*modB*) causing six α -subunit type amino acid substitutions as well as *EGFP* gene [75]. The gene products, modified HexB (*modB*, a homodimer of the modified β -subunits) different from the wild-type HexB, can recognize negatively charged artificial substrates and bind to GM2A to exhibit GM2-degrading activity. We transduced the MSCs derived from *Hexb*^{-/-} mice (SD MSCs) with the MSV-*modB*, resulting in restoration of HexA-like activity and reduction of the accumulated GM2 and GlcNAc-oligosaccharides. The *modB* was also secreted from the transduced SD MSCs. In addition, we performed intraventricular engraftment of the transduced SD MSCs expressing *modB* into the brain of *Hexb*^{-/-} mice. As a result, the injected transduced SD MSCs expressing HexA-like activity and EGFP were observed in periventricular region of the brain (Figure 1). Reduction of the immunoreactivity towards natural substrates including GM2 and GlcNAc-oligosaccharides were also observed around the periventricular region of *Hexb*^{-/-} mice brain (Figure 2). These results suggest that genetically modified MSCs can be utilized as a brain-directed donor cells for *ex vivo* GT for LSDs involving neurological manifestations, including Tay-Sachs and Sandhoff diseases.

Lee J-P. *et al.* demonstrated intracranial transplantation of neural stem cells (NSCs) delayed onset, improved motor function, reduced GM2 storage and prolonged life span in the *Hexb*^{-/-} mice partly due to the cross correction effect of the Hex isozymes secreted from NSCs. Human NSCs derived directly from the CNS and secondarily induced from embryonic stem

(ES) cells also demonstrated a broad repertoire of potentially therapeutic actions, which are expected to be applied for the treatment of neurodegenerative diseases [95]

5. Conclusion

A number of preclinical and therapeutic approaches for GM2 gangliosidoses, including stem cell therapy, substrate deprivation therapy, gene therapy, and enzyme replacement therapy, are being examined and evaluated with disease model mice, although there is no effective therapy for treatment of the patients with GM2 gangliosidoses at present. However, according to the preclinical results obtained by using animal disease models, CNS-directed *in vivo* gene therapy utilizing recombinant viral vectors and *ex vivo* gene therapy based on the cross-correction by transduced autologous and heterologous stem cells are promising for development of novel therapies for LSDs associated with neurological abnormalities, including GM2 gangliosidoses. Improvement of these GTs and their combination with other clinical approaches will facilitate the development of efficient therapies for neurodegenerative disorders caused by neuroinflammation and gliosis.

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Applications: Others

Gene Therapy Perspectives Against Diseases of the Respiratory System

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Additional information is available at the end of the chapter

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1. Introduction

Gene therapy uses a variety of techniques as the introduction of a normal allele of a gene in cases where the cell does not express the gene or in other cases where the gene is under-expressed. In order to achieve effective gene therapy for a specific gene in a certain type of cells a lot of work is needed. More specifically the following steps are essential: 1. Isolation of target gene, 2. Development of a specific gene vector, 3. Specification of the target cell, 4. Definition of route of administration, and 5. Identification of other potential uses of the gene.

The value of gene of gene therapy is often discussed, especially in some diseases who have a known protein defect and the protein itself can be produced in a large scale and could then be administered to the patient. Genetic engineering could be beneficial in the production of the target protein. Nevertheless, the infusion of the protein is not curative, because of the half-life of the protein itself and the growth factors that are essential.

In order to isolate a specific gene, it is essential to produce a cDNA library that contains the total number of unique genes expressed in a specific tissue. The DNA contained in a cDNA library is not genomic, therefore it contains only the encoding sequences of the DNA.

The standard procedure of the construction of a cDNA library includes 1) isolation of the total amount of mRNA that is produced in the target cells, 2) Hybridization using a multi-T promoter, 3) Synthesis of complementary DNA (cDNA) to the mRNA prototype using the enzyme reverse transcriptase, 4) Degradation of the mRNA by the means of an alkali, 5) Synthesis of the second DNA strand using nucleotides and the enzyme DNA polymerase.

The cDNA library contains only the exons of the genes that are expressed in the specific tissue; therefore the cDNA can show the activity of the studied tissue.

As soon as the isolation of the gene that is to be administered to the patient is achieved, an appropriate vector is needed in order to deliver the gene to the target cells. The most important vectors that are generally used in gene therapy applications in order to perform transfection of the targeted cellular population are:

1. Plasmids which are well-tolerated and safe, but transfer towards the nucleus is not so easy
2. Adeno-virus which may transfect differentiating as well as stable cells and have a very good percentage of transfection, but is not inserted in the nucleus and there is a possibility of reaction against the adeno-virus
3. Retro-virus which are inserted in the genome and are stable during transport, but they can only be used in transfection of multiplying cells
4. Lenti-virus which is a subtype of retro-virus that may be inserted in stable cells and it is quite stable during the procedure
5. AAV (adeno-associated-based vector) which is inserted in the genome, is quite stable during the procedure and stable cells can be transfected as well, but only 4,7 kb can be inserted and there is a possibility of mutations
6. Liposomes – Oligonucleotides (ODN-based) which are very easy to use, selective for the endothelium, special alterations can improve the availability and reduce toxicity

The target cell has to be defined carefully in order to achieve the best curative result. In the case of gene therapy in the lung, the airway epithelia or even the lung vasculature may be efficient cellular targets.

The route of administration has to be defined so as the target gene is transported to the target cells in order to perform the transfection of the target tissue cells.

The use of a target gene in the therapy of a certain condition of the lung does not exclude a possible use of the gene in another therapeutic strategy, where there is a similar pathophysiology (e.g. inflammation). Therefore, the identification of other potential uses of the target gene is always important.

2. Gene therapy in cystic fibrosis

Gene therapy is still far from becoming a curative treatment for cystic fibrosis (CF). Despite the outstanding technological and medical progress there is still number of interesting genetic, biological, pharmaceutical and ethical problems. Only when these issues are to be solved will gene therapy become an option for the treatment of CF.

As for the biology of CF, the cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in airway epithelia, on the luminal side of the plasma membrane, where it plays

an important role as a phosphorylation-regulated Cl^- channel and a regulator of channels and transporters [1, 2]. More specifically, the activation of CFTR results in a parallel inhibition of the epithelial Na^+ channel (ENaC), which is lost when CFTR is absent or not functioning. There is a so called “low volume” hypothesis, which suggests that a loss of Cl^- secretion and an increase in Na^+ absorption reduce the thickness of the airway surface liquid (ASL), thus impairing mucociliary clearance [3]. Moreover, a reduction in the secretion of bicarbonates (mediated by the CFTR) might affect the hydration of the secreted mucus, thus altering its physical properties [4]. CFTR is also expressed in submucosal glands in the airways, which mainly participate in host defence. A loss of CFTR function in duct-lining serous cells prevents the secretion of mucus and anti-microbial factors by submucosal glands [5].

Since the discovery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in 1989, it was thought that scientists could prevent or delay the onset or even the progression of lung disease by using gene transfer. Although loss of CFTR function may affect a great number of different cells and tissues, progressive lung disease is responsible for the rates of morbidity and mortality. Therefore, the efforts of gene therapy have focused so far on gene transfer to the airways. CFTR is expressed in various epithelial cells in the lumen and in submucosal glands of the airways, where the mRNA is expressed [6, 7].

The fact that CF is an autosomal recessive disease lead to the idea that the delivery of a CFTR cDNA to the airway epithelium with a viral or non-viral vector could have beneficial effect. The delivery method could be either direct instillation or aerosol delivery. Furthermore, early studies indicated that the transfection of 6–10% of CF epithelia generated wild-type levels of chloride transport *in vitro* [8].

The selection of targets cells for gene therapy in CF is still controversial. The available strategies suggest correcting cells of the surface epithelium, the submucosal glands, or both [9, 10, 11]. The CFTR is expressed in the airways, including ciliated cells within the surface epithelium and a subpopulation of cells in submucosal gland ducts and acini. There are several epithelial cell types in the lung that seem to have progenitor functions, thus allowing long-term correction if these cells are targeted with selected vectors [12]. Experiments from several species and model systems have revealed potential progenitor populations, including: basal cells [13] and non-ciliated columnar cells of the airways [14, 15], submucosal gland epithelia [16], Clara cells [17] and alveolar type II cells in the distal lung.

Many viral and nonviral vectors have been tested for their usefulness in CF gene therapy. Adenoviral (AV) vectors have as a great disadvantage their low transduction efficiency of human airway epithelia and by their induction of strong immune responses [18]. In contrast adeno-associated viral (AAV) vectors may lead to long-term gene transfer and expression in bronchial epithelia of rabbits and nonhuman primates.

In addition to the DNA viruses, AV and AAV, various RNA viruses have been investigated for uses in airway gene transfer. Murine parainfluenza virus type 1, human respiratory syncytial virus (RSV) and human parainfluenza virus type 3 (PIV3) can effectively transfect airway epithelial cells by attaching to sialic acid and cholesterol [19], which are found on the

apical surface of these cells. These viruses replicate in the cytoplasm and do not seem to cause mutagenesis during the insertion in DNA. Although RSV and PIV3 are human pathogens, SeV, the only RNA virus for which efficiency has been assessed *in vivo*, is not. However, gene expression mediated by recombinant SeV-based vectors needs repeated administration, which does not seem feasible because of the development of neutralizing antibodies against the vector itself [20].

Lentiviral (LV) vectors derived from human immunodeficiency virus type 1 (HIV-1) and feline immunodeficiency virus (FIV) are integrating retroviruses which can be adequately utilized to achieve efficient transfection of airway epithelia [21].

Among the many nonviral gene therapy vectors investigated so far, GL67 ([Cholest-5-en-3-ol(3b)- 3-[(3-aminopropyl)4-[(3-aminopropyl)amino]butyl] carbamate]) has emerged as a promising lipid for efficient lung transfection [22].

Finally, mRNA-based nonviral gene transfer is a new strategy in order to express the CFTR in target cells [23]. By the use of mRNA instead of plasmid DNA as the transgene, transfection efficacy depends on the cytoplasmic expression machinery. However, when compared to DNA, much less is known about immune responses to RNA, although responses to both seem to be mediated by Toll-like receptors (TLRs).

As a chronic, lifelong disease, CF will be best treated with a continuous level of CFTR expression. This could be achieved either by repeated application or with a long-duration expression system. Viral vectors, which are mainly used in gene therapy appear difficult to administer repeatedly [24], in contrast to synthetic approaches [25].

The use of genomic DNA that contains all the control elements that allow gene expression at physiological levels has been utilized [26]. Extensive knowledge of the critical regulatory elements in the CFTR locus is required.

The CFTR gene maps at 7q31.2 and the expression is regulated during development and in different tissues. The CFTR locus is in connection with genes with different tissue-specific expression profiles, suggesting the presence of specific control promoters and insulators. Nuclear localization studies of CFTR and its adjacent gene loci in humans and mice demonstrate that different chromatin regions behave independently, depending on their expression profiles [27].

2.1. Applications of RNA interference to treat CF

The recent knowledge in the field of small interfering RNAs has led to the development of applications in relevance to CF. The RNAi technology has been used in order to identify gene products that contribute to steps in wild-type and mutant CFTR production and action [28]. Therefore, there is a possibility that RNAi-based strategies could be developed to increase the expression of $\Delta F508$ CFTR, to rescue $\Delta F508$ CFTR from proteosomal degradation or prolong its action on cell membrane. Similarly, targeting other cellular pathways, such as the inflammatory process, might lead to the reduction of symptoms. A significant obstacle

to overcome is the identification of methods to efficiently deliver RNAi to differentiated airway epithelia.

2.2. Lung tissue engineering

Lung transplantation is currently the only definitive treatment for end-stage CF lung disease. However, the availability of donors is limited and the survival of transplantation is hardly 10–20% at 10 years [29]. Recently, two groups independently used similar tissue-engineering strategies to develop an autologous bioartificial lung that may begin to help overcome the limited availability of donor tissues [30, 31]. Evidence for gas exchange within the resulting grafts was demonstrated. Following the development of this technology, the *ex vivo* correction of patient-derived cells and the transplantation of these cells could lead to the cure of the disease. Although these initial results are very exciting, several steps need to be further optimized before long-term tissue-engineered lung function can be used in patient applications [32].

3. Gene therapy in Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease is a disease characterised by the presence of airflow obstruction generally progressive due to chronic bronchitis or emphysema and may be partially reversible. COPD is the 4th leading cause of death in the United States. In 2000, the WHO estimated 2.74 million deaths worldwide from COPD. In-patient hospitalization and emergency department care accounts for >73% of this cost COPD costs \$1,522 per person per year (3 times asthma costs) [GOLD 2008].

Tobacco smoke is by far the most important risk factor for COPD worldwide. Other important risk factors are occupational exposure, socioeconomic status and genetic predisposition [33]. Thus, investigating into copd and into management possibilities is of high importance in order to provide the essential help to the patients. The currently used drugs can manage effectively the main symptoms of copd and may control the symptoms of this condition.

To date, there are no effective treatments for emphysema, nor are there efficient clinical management strategies. Novel approaches using gene therapy and stem cell technologies may offer new opportunities. However, this will remain almost entirely dependent on a more thorough understanding of the pathogenesis of COPD [34]. Currently, the most accepted theory for the development of COPD is protease/ antiprotease imbalance similar to emphysema due to hereditary 1-antitrypsin deficiency [35]. Newer studies [36] have shown that the pathogenesis of COPD involves not only elastases, but also collagenases and gelatinases. Experimental models [37] have suggested a role for 1-antitrypsin and secretory leuko-protease inhibitor in the treatment of this disorder. However, there is still need for a convincing study proving the concept of antiprotease treatment for COPD and emphysema [38] Neutrophils are a major source of proteases and reactive oxygen, so gene therapy could also target adhesion molecules for neutrophils to reduce their accumulation into the lung parenchyma.

3.1. A1-antitrypsin deficiency

A1-antitrypsin (AAT), is a major anti-protease serum protein, counteracting the effects of neutrophil elastase and other pro-inflammatory molecules released at sites of lung inflammation [39]. There are not effective treatments using protein therapy so gene therapy is being evaluated as an alternative approach.

Early studies in cotton rats using first-generation Ad vectors resulted in detection of AAT in bronchoalveolar fluid for only 1 week post-administration [40]. Cationic liposomes have also been used to express human AAT in the rabbit lung following aerosolisation [41]. Recombinant AAV vectors are being evaluated for more persistent expression of therapeutic serum levels of human AAT in murine and non-human primate models following intramuscular injection [42]. A1-antitrypsin deficiency is a pulmonary disease with an underlying single gene defect and a target for gene therapy. One specific treatment for AAT deficiency available is the administration of AAT intravenously, but only 2–3% of the infused AAT actually reach the lungs. Another method of administration is the inhalation of nebulized AAT powder or aerosolized AAT solution [43]. However, the treatment by the means of an alternative therapy, namely gene therapy, provides long term solution [44]. Several vectors containing cDNA of AAT have been constructed for treating AAT deficiency diseases. These vectors are retroviral [45], adenoviral [46] and adeno-associated viral [47]. Besides this, AAT gene can also be transferred by liposomal vectors [48]. First clinical trial has demonstrated that AAT gene could be transferred in humans [49]. Patients with AAT deficiency received a single dose of non-viral cationic liposome. Protein Gene Therapy for Alpha-1-Antitrypsin Deficiency Diseases was detected in nasal lavage fluid, with maximum levels on fifth day, which is approximately one third of the normal levels. The retroviral vector containing cDNA of human AAT with constitutive promoter have also been used as a delivery system. The disadvantage of retroviral vector system is that transgene expression is low. The adenoviral vectors containing human AAT cDNA have been delivered to different organs and cells [50]. Results in vitro demonstrated that human alpha-1-antitrypsin was synthesized as well as secreted. The adenoviruses are pathogenic in nature as well as immunogenic, therefore they have limited applications in treating AAT deficiency diseases. Recombinant adeno-associated viral vectors have been most successful delivery system so far, as they are capable of achieving therapeutic levels of AAT [51], and are less likely to induce an inflammatory response than adenoviral vectors. These viral and non-viral vectors showed advantages as well as disadvantages in curing AAT deficiency diseases. Among tested rAAV serotypes, the rAAV8 was found to be more powerful gene therapy vector for treating lungs and liver diseases [52]. Newly developed AAV vector looks promising for treating AAT deficiency diseases.

4. Gene therapy in asthma

Asthma is a disorder defined by certain clinical, physiological and pathological characteristics. Asthma is a chronic inflammatory disorder of the airways associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest

tightness, and coughing, usually associated with widespread, but variable, airflow obstruction that is often reversible either spontaneously or with treatment [GINA 2000]. Since its pathogenesis is not clear, this definition is descriptive and inclusive of different phenotypes that are being increasingly recognized. Worldwide, 300 million people are supposed to be affected by asthma [53]. It appears that the global prevalence of asthma ranges 1–18% of the population in different countries. The WHO has estimated that 15 million disability-adjusted life-yrs are lost annually due to asthma, representing 1% of the total global disease burden [54]. Annual worldwide deaths from asthma have been estimated at 250,000 and mortality does not appear to correlate well with prevalence.

The best treatment of asthma is inhaled corticosteroids and bronchodilators, for the majority of asthmatic patients [55]. Gene therapy could bring some benefit for asthmatic patients with uncontrolled asthma who require high doses of corticosteroids and for patients with corticosteroid-resistant asthma. The target of gene therapy in bronchial asthma could be the overexpression of T-helper (Th) type 1 cytokines that influence the Th2 cytokine reactions [56]. Moreover the overexpression of IL-12 also restored local antiviral immunity, which is impaired in a Th2-dominated environment particularly during exacerbations of bronchial asthma due to viral infections [57]. Another study [58], examined the gene transfer of IFN- γ that is a very interesting mediator in the airway hyper-responsiveness. Furthermore, another newer study [59] has shown that the transfer of the glucocorticoid receptor gene *in vitro* mediated the inhibition of nuclear factor- κ B activities even in absence of exogenous corticosteroids, and the authors suggested that this approach could restore corticosteroid sensitivity in patients.

5. Gene therapy in lung cancer

Lung cancer is the most common cancer worldwide, it is responsible for 12.4% of new cases of cancer in 2002. The overall mortality is 87% and 5-year survival is estimated to range from 15% in USA to 8.9% in developing countries [60]. It ranks first as the cause of death and it is responsible for 1.18 million deaths in 2002 and it is accounted by the World Health Organization for 18.4% of all cancer deaths by 2015. Non-small cell cancer (NSCLC) accounts for approximately 85% of lung cancers [61].

Even if there have been a lot of advances in surgery, radiation and chemotherapy, the 5-year survival for lung cancer remains poor. There is now great interest in gene therapy approaches for thoracic malignancies. Lung cancer is usually metastatic at the time of diagnosis and systemic therapy is needed rather than local therapy.

Gene therapy for thoracic malignancies represents a therapeutic approach that has been evaluated in clinical trials for the last two decades. Using viral vectors or antisense RNA, strategies have included induction of apoptosis, suicide gene expression, cytokine based therapy, various vaccinations and adoptive transfer of modified immune cells.

5.1. Clinical trials

5.1.1. Replacement of tumor suppressors

The goal of this strategy is to use a gene vector in order to encode a tumor-suppressor protein in tumor cells that is mutated or absent in the majority of lung cancers.

Tumor-based p53 therapy: It has been shown that the replacement of the normal p53 tumor suppressor gene in tumor cells induces rapid cell death by studies in cellular and animal models. In several early-phase clinical studies the strategy of the restoring the wild-type p53 expression in lung tumor cells was studied. The first study to demonstrate the feasibility of tumor suppressor gene replacement mediating tumor regression was a phase I study in which a retrovirus vector carrying wild-type p53 was administered to 7 patients with lung cancer with direct intra-tumoral injection. There was evidence of increased apoptosis in 6 patients and tumor regression in 3 patients [62]. Other phase I studies of p53 replacement with adenoviral vectors resulted in few partial responses and several patients with stabilization of disease [63, 64, 65]. Weill et al. delivered Ad.p53 to obstructive lesions endobronchially and they had several partial responses [66]. In another large phase I study of Ad.p53 gene transfer that was delivered intra-tumorally in combination with chemotherapy, it was shown that there was increased apoptosis in transduced tumors when examined histologically [67]. A single-arm phase II study of intratumoral Ad.p53 in combination with radiation showed tumor regression in 63% (12 of 19) and was well tolerated [68]. But in another phase II study there was no difference in response rates for Ad.p53/chemotherapy-treated lesions for primary tumor lesions versus lesions treated with chemotherapy alone and this showed that Ad.p53 provided little local benefit over chemotherapy [69]. Keedy et al. delivered repeatedly Ad.p53 by bronchoalveolar lavage (BAL) to patients with bronchoalveolar carcinoma. It was shown that this delivery resulted in transient expression of p53 in 19% (3 of 16) of patients, 2 of the 3 patients achieved stable disease. It was suggested that BAL could be used for adenoviral delivery, but toxicity was a serious issue with this approach [70]. Guan et al. delivered Ad.p53 alone or in combination with bronchial artery instillation (BIA) of chemotherapy (fluorouracil, navelbine or cisplatin). The delivery of Ad.p53 was performed via direct percutaneous delivery or via BIA. There was 47% response rate in the combination group and an improvement in time to progression when compared with BAI alone [71]. The Adp53 has been approved for usage in neck and head cancers in China, but there are not any trials using Ad.p53 in lung cancer in USA. Vanchani et al. believed that there is a strong issue with the application of this method in lung cancer (especially treating endobronchial lesions) as there is no bystander effect in combination with low transfection efficiency of adenoviral vectors [72].

FUS1 Replacement: FUS1 is a novel tumor suppressor gene that was identified in human chromosome 3p21.3 region where allele losses and genetic alterations occur for some human cancers. In most premalignant lung lesions and lung cancers the expression of FUS1 protein is absent. It was shown that wt-FUS1 function was restored in 3p21.3-deficient non-small cell lung carcinoma cells and this function inhibited tumor cell growth by induction of apoptosis and alteration of cell cycle kinetics [73].

Gene-Modified Dendritic Cell-Based Vaccination: Dendritic cells (DC) are the most potent antigen presenting cells in the immune system and they have been used for vaccination as vaccine vehicles. They have been used in two ways. The first one is to modify DC *ex vivo* with chemokines or cytokines and inject them directly into tumors and then they take antigen and induce immune response. The second one is to load immature, phagocytic DC with antigen with the aid of purified protein, cell extracts, mRNA and gene vectors and after that they inject these DC subcutaneously.

Ad.p53: p53 protein: It is proposed as a tumor antigen for vaccines as mutant p53 exists in very high levels in tumor cells and has more prolonged half-time than normal cells. p53-based gene therapy (p53 transduced DC) with standard chemotherapy showed promising results [74]. In a phase I trial, 29 patients with small cell lung cancer were vaccinated with DC transduced with Ad.p53 and the result was 1 patient with partial response and 7 cases with stable disease. Besides, out of the 21 patients that received a second line of chemotherapy, there was 62% response rate much higher from the rate that it is known for the second line therapy in small cell lung cancer. There was also a better survival (12.1 months instead of 9.6 months) in patients that showed an immune response to vaccination.

CCL21: CCL21 is a CC chemokine which is expressed in high levels in high endothelial venules and T cell zones of spleen and lymph nodes and also it attracts mature DC, naive T cells and induces T-cell activation [75]. Preclinical data showed that there was potent activity against lung cancers when DC transduced with CCL1 were injected into tumors.

Gene-Modified Tumor Cell-Based Vaccination: Killed tumor cells (usually irradiated) have been injected into patients as vaccines against recurrent cancers for many years with partial successful results.

Transforming growth factor $\beta 2$ antisense vector modified cells: It is known that increased levels of transforming growth factor (TGF- $\beta 2$) are associated with greater immunosuppression and poorer prognosis in patients with NSCLC. Preclinical studies showed that the delivery of an antisense gene to TGF- $\beta 2$ to *ex vivo* tumor cells inhibited cellular TGF- $\beta 2$ expression and resulted in increased immunogenicity when these tumor cells were administered as a vaccine. In a phase II trial this method of vaccination with irradiated tumor cells modified with a TGF- $\beta 2$ antisense vector (belagenpumatucel-L) was evaluated. There was better survival (dose-related) with minimal toxicity. Besides there were different immunologic end points such as increased levels of cytokines (INF- γ , interleukin-6,interleukin-4) and increased levels of antibody production to vaccine HLAs. In a trial, 21 patients received belagenpumatucel-L at a single dose [76]. It was shown that 70% of cases were stable, but there was no complete or partial response. There is an ongoing phase III trial in which this vaccination is evaluated.

Tumor cells modified to secrete granulocyte-monocyte colony stimulating factor(GVAX): Granulocyte-monocyte colony stimulating factor (GM-CSF) is a cytokine that is involved in the maturation and proliferation of myeloid progenitor cells and stimulates proliferation, maturation and migration of DC and that leads to induction of T-cell immune responses against cancer. There are preclinical studies in which the transfection of tumor cells with the

GM-CSF gene has led these cells to induce antitumor immune responses. The clinical trials in lung cancer started using a vaccine platform with intradermal vaccination of irradiated autologous tumor cells that were virally engineered to secrete GM-CSF [77, 78]. In the first trial of cases of metastatic NSCLC, GM-CSF was transduced into autologous tumor cells with the aid of adenoviral vector before irradiation and vaccination. There were a few clinical responses with a strong immune response. A delayed hypersensitivity reaction to irradiated, autologous nontransfected tumor cells was observed in patients. Nemunaitis J, et al. used a similar strategy in early-stage and late-stage patients and they showed that there were several clinical responses with similar immunologic outcomes [79]. In another trial, Nemunaitis J, et al. used a vaccine of unmodified, irradiated autologous tumor cells mixed with a GM-CSF-secreting bystander cell line. The vaccine GM-CSF secretion was higher than with the autologous vaccine, but the frequency of vaccine site reactions, tumor responses and survival were less favorable with the bystander vaccine [80]. Finally, the GVAX approach was not used more in lung cancer because the results were not satisfied and now only studies in pancreatic cancer are going on.

a(1,3)Galactosyltransferase: The gene that encodes a(1,3) Galactosyltransferase is not active in humans and it is functional in other mammalian cells. The major mechanism of hyperacute rejection of xenotransplants is the production of anti-a Gal antibodies in humans. Morris J et al. used allogeneic NSCLC tumor cells that were retrovirally modified to express aGT. It was shown that 6 of 17 patients, that received intradermal treatments, had prolonged stable disease [81].

B7.1/HLA vaccination: B7.1 is the one that costimulates T cells during priming by an antigen-priming cell. In a phase I trial of 19 patients with advanced NSCLC, treatment with an allogeneic lung cancer cell line vaccine transfected with B7.1, HLA-A1 and HLA-A2 was done. There was one partial response and 5 cases with stable disease. In the 6 responders, the CD8 T cell titers to tumor cell stimulations were elevated steadily till 150 weeks after therapy [82]. A phase II trial is ongoing in patients with stage IIIB/IV who fail after the first line chemotherapy.

5.1.2. Vaccines

MUC-1 vaccination: MUC-1 is a tumor-associated mucin-type surface antigen normally found on epithelial cells in many tissues. In cases of lung cancer the targeting of MUC-1 has been used in a lot of ways with gene and non-gene therapy approaches. Ramlau R et al. in their 2 arm phase II trial with 65 patients with IIIB/IV NSCLC used a vaccinia virus containing the coding sequences for MUC1 and IL-2 (TG4010). The patients that participated in the trial had MUC-1 antigen expression on the primary tumor or metastases. In the 1ST arm (44 patients), combination therapy with TG4010 and cisplatin/vinorelbine was given, and in the 2nd arm TG4010 monotherapy was given followed by combination therapy at progression. In the 1st arm there was partial response in 29.5% and survival rate of 53% for the 1st year. In the 2nd arm, two of the 21 patients had stable disease for more than 6 months with monotherapy of TG4010 and this arm was terminated early as the results were not satisfied. There were MUC1-specific responses for 12 of 21 patients with stable disease or partial response.

Disease control was observed for 4 of 5 patients. The existence of MUC1 specific responses was translated to longer time to progression and better overall survival [83].

L523S vaccination: L523S is an immunogetic lung antigen that is expressed in 80% of lung cancer cells. Nemunaitis et al. in a phase I study, they gave two doses of intramuscular recombinant DNA followed by two doses of Ad.L523S (given 4 weeks apart) to 13 patients with early stage NSCLC(stage 1B,IIA and IIB). The authors found that only 1 patient showed a L523S-specific antibody response [84].

5.2. Antisense therapy

This technology is able to downregulate a lot of molecules that promote lung cancer tumor growth. There are 3 trials with antisense therapy. In the first trial aprinocarsen was used. Aprinocarsen is an oligonucleotide that binds to mRNA for protein kinase C- α and inhibits its expression. It was demonstrated that this molecule was safe in patients with lung cancer and it was characterized by modest activity in combination with chemotherapy [85]. In another trial with chemotherapy with or without aprinocarsen as first line therapy, it was shown that there was no better survival but with some toxicity as well [86]. In phase I studies it was shown that few patients had prolonged stable disease and 1 patient had response with the administration of Raf antisense molecules [87]. In patients with lung cancer in two phase II studies, these molecules did not show any antitumor activity [88]. Next, in other trials, the authors used Bcl-2, an apoptotic inhibitor which is overexpressed by many tumors and especially by 80-90% of SCLCs; the existence of this inhibitor means increased resistance to chemotherapy. In two trials there were encouraging results [89, 90], but in another trial of standard chemotherapy with or without a bcl-2 antisense oligonucleotide (oblimersen) more hematologic toxicity and worse overall survival was observed in the experimental arm [91].

5.3. New directions

The trials that have been made about gene therapy in lung cancer, preclinically and clinically, have demonstrated intermittent efficacy. The technology of gene transfer is promising but it is not easy to transduce more than a small number of tumor cells. This is a very important issue especially with approaches that they do not have bystander effects. It is very important to create vectors that they are able to induce long term in vivo expression as lentiviruses and AAVs.

Another interesting strategy is the immune-gene therapy, which requires gene transduction for stimulating an endogenous immune response and in this way a bystander effect is generated. There are some encouraging approaches with gene therapy to stimulate anti-tumor responses by delivering immunostimulatory cytokines or by administering a vaccine.

There is another important field of creating adoptive transfer of gene-modified autologous lymphocytes that are modified ex vivo by using lentiviruses or retroviruses. This approach is directed against mesothelioma and lung cancer cells.

6. Conclusion

Gene therapy is a very promising tool for the respiratory clinician and a few clinical trials have been performed. All these trials have shown safety but intermittent efficacy. Gene therapy for pulmonary diseases has not yet reached the point of clinical practice. But we can say that this tool will find a very interesting role in our efforts for treating respiratory diseases in the future.

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Gene Therapy in Critical Care Medicine

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Additional information is available at the end of the chapter

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1. Introduction

Critical care medicine is directed toward patients with a wide spectrum of illnesses. These have the common denominators of marked exacerbation of an existing disease, severe acute new problems, or severe complications from disease or treatments. In recent years has been an explosion of evidence based medicine with improvement in outcome, however there are several conditions in critical care patients that maintains a high morbidity and high mortality that is necessary to be addressed [1]. Of these, severe sepsis and the acute respiratory distress syndrome (ARDS), including acute lung injury (ALI) (syndromes consisting of acute respiratory failure associated with pulmonary infiltrates due to intra- or extra-pulmonary diseases) are two important conditions that have increased mortality in critical care units around the world [2, 3].

In 1991, a Consensus Conference of the American College of Chest Physicians and the Society for Critical Care (ACCP-SCCM) introduced the term systemic inflammatory response syndrome (SIRS) as the presence of at least two of four clinical criteria: body temperature more than 38°C or less than 36°C, heart rate more than 90 beats per minute, respiratory rate more than 20 breaths per minute or hyperventilation with PaCO₂ less than 32 mmHg, white blood cell count more than 12000/mm³, less than 4000/mm³ or with more than 10% immature neutrophils [4]. In 2001, a new consensus suggests that other signs and symptoms could reflect the clinical response to infection, including: fever/hypothermia, tachypnea/respiratory alkalosis, positive fluid balance/edema, general inflammatory reaction, altered white blood count, increased biomarkers (C-reactive protein, IL-6, pro-calcitonin), hemodynamic alterations, arterial hypotension, tachycardia, increased cardiac outflow/low systemic vascular resistance/high venous saturation O₂, altered skin perfusion, decreased urine output,

hyperlactacemia, signs of organ dysfunction, hypoxemia, coagulation abnormalities, altered mental status, hyperglycemia, thrombocytopenia, disseminated intravascular coagulation, altered liver function, intolerance to feeding [5].

Systemic inflammatory response syndrome can result from diverse etiologies, including, but not limited to infectious, trauma, pancreatitis, ischemia-reperfusion injury, and burns [6]. Sepsis is defined as the presence of infection and some of the listed signs and symptoms of SIRS, whereas severe sepsis is defined as sepsis associated with organ dysfunction and shock septic as severe sepsis with hypotension, despite adequate fluid resuscitation [7].

Over 18 million cases of severe sepsis occur each year. The number of severe sepsis cases is set to grow at a rate of 1.5% per year from the annual incidence of 3 cases per 1000 of the population in 2001 [8, 9]. Sepsis is a major cause of mortality throughout the world, killing approximately 1400 people every day, being as high as an additional fifty per cent as deaths are often attributed to complications from cancer or pneumonia, and not related to sepsis [10]. Death is common among sepsis patients, with around 28-50% of patients dying within the first month of diagnosis [11-13]. Sepsis impacts the lives of many people, including the patient and their families, in addition to doctors, nursing and care staff. The intense demands made on hospital staff, equipment and facilities to treat septic patients places a significant burden on healthcare resources, accounting for 40% of total ICU expenditure [10]. Each year the cost of treating septic patients increases and is as high as 7.6 billion euro in Europe [10] and 17.4 billion euro in the USA [8].

One common complication of SIRS and sepsis is acute lung injury/adult respiratory distress syndrome (ALI/ARDS). According to a Joint North American European consensus committee (NAECC), ARDS is defined as an inflammatory process in the lungs with acute onset of respiratory failure, new bilateral pulmonary infiltrates on frontal chest radiograph or computed tomography, absence of left ventricular failure (clinically diagnosed or a pulmonary artery occlusion pressure $<18\text{mmHg}$) and hypoxemia with a ratio between the partial pressure of arterial oxygen and the fraction of inspired oxygen ($\text{PaO}_2/\text{FiO}_2$ ratio) of $\leq 27\text{ kPa}$ independent of the level of positive end-expiratory pressure (PEEP) [14]. ALI is defined by the same criteria except that the $\text{PaO}_2/\text{FiO}_2$ ratio is between 27 kPa and 40 kPa [14-16]. Sepsis is the most common cause of ALI/ARDS and also the most common cause of death after patients develop ALI/ARDS [17]. The incidence of ALI/ARDS is estimated to be 20 to 50 cases per 100000 person-year, with approximately 18% to 25% of cases meeting oxygenation criteria for ALI but not for ARDS [18, 19].

The reported rate of mortality from ARDS ranges from 31% to 74% depending on the characteristics of patients, with most deaths occurring as a consequence of multiple organ failure and sepsis [18, 19]. ALI has a significant lower crude hospital mortality (32%) compared with those with ARDS (57.9%) [20]. Crude estimates of the health care costs associated with ALI/ARDS may exceed 5 billion dollars per year in the United States alone [21].

2. Physiopathology of sepsis

Microorganisms express macromolecular motifs, named pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), flagellin, double-stranded RNA and CpG DNA [22]. These molecules are recognized by the immune system through a family of trans-membrane or intra-cytoplasmic receptors, the pattern recognition receptors (PRRs), classified in three general families: a) Toll-like receptors (TLRs); b) NOD-like receptors (NLRs); and c) RIG-I-like receptors (RLRs) [22].

The TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain [23]. Based on their primary sequences, TLRs can be divided into several subfamilies, each of which recognizes related PAMPs: the subfamily of TLR1, TLR2 and TLR6 recognize lipids, whereas the highly related TLR7, TLR8, TLR9 recognize nucleic acids. TLR4 recognizes a very divergent collection of ligands [24]. The NLR proteins are implicated in the recognition of bacterial components. Proteins in this family possess LRRs that mediate ligand sensing: a nucleotide binding oligomerization domain (NOD) and a domain for the initiation of signaling such as CARDs, PYRIN of baculovirus inhibitor of apoptosis repeat (BIR) domains [25]. The retinoic-acid inducible protein-1 (RIG-I) is an INF-inducible protein containing CARDs and a DExD/H box helicase domain and has been identified as a cytoplasmic detector in viral infection in the TLR3 independent manner [26]. In addition to the numerous exogenous pathogen-derived ligands that activate different TLRs, endogenous TLR ligands have been identified, including hyaluronic acid, high mobility group box-1 (HMGB1) and heat shock proteins (HSPs), termed as damaged-associated molecular patterns (DAMPs). During tissue injury or proteolysis, extracellular matrix components undergo cleavage, exposing moieties that can act as ligands for TLRs and therefore initiating TLR-induced signal transduction [27].

The PAMP/PPR interaction leads to immune cell activation with initiation of microbe-killing systems, production and secretion of pro-inflammatory cytokines and chemokines, enhanced expression of co-stimulatory receptors essential for efficient T cell activation, production of arachinoid acid metabolites and initiation of extrinsic coagulation cascade [28-33]. The activation of the TLR signaling originated from the cytoplasmic Toll/IL-1 receptor (TIR) domain requires the association with the TIR domain-containing adaptor protein, MyD88. With ligands binding, MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs through interaction of the death domains of both molecules. IRAK-1 activated by phosphorylation then associates with TRAF6, finally leading to activation of MAP kinases and NF κ B. Additional modes of regulation for these pathways include TRIF-dependent induction of TRAF6 signaling by RIP1 and negative regulation of TIRAP mediated downstream signaling by ST2L, TRIAD3A and SOCS1. MyD88-independent pathways induce activation of IRF3 and expression of interferon- β . TIR-domain containing adaptors such as TIRAP, TRIF and TRAM regulate TLR-mediated signaling pathways by providing specificity for individual TLR signaling cascades [28-33].

The interaction of PAMs with NRL recruits the receptor-interacting protein-2 (RIP2) kinase activating NF κ B and MAPK kinases. A number of the NRL molecules have been shown to form a complex with caspase-1 and the adaptor molecule apoptosis associated speck-like protein containing CARD (ASC) termed inflammasome. The central effector molecule of the inflammasome is the cysteine protease caspase-1 that, upon activation cleaves cytosolic pro-IL-1 β , pro-IL-18 and pro-IL-33 to their active forms enabling them to be secreted into the extracellular/systemic compartments [34]. The important fact is that NRLs and TLRs may synergize. T-cell subgroups are modified in sepsis. Helper (CD4⁺) T-cells can be categorized as type 1 helper (Th1) or 2 (Th2). Th1 cells generally secrete pro-inflammatory cytokines such as tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β); Th2 cells secrete anti-inflammatory cytokines such as IL-4 and IL-10, depending on the infecting organism, the burden of infection and other factors during sepsis may also induce apoptosis of lung and intestinal cells [35]. Activated helper T cells evolve from a Th1 phenotype, producing pro-inflammatory cytokines, to a Th2 phenotype producing anti-inflammatory cytokines [35]. In addition, apoptosis of circulating and tissue lymphocytes (B cells and CD4⁺ T cells) contributes to immunosuppression [36]. The increased pro-inflammatory cytokines, activated B cells and T cells and circulating glucocorticoid levels causes apoptosis in septic patients [37]. Increased levels of TNF- α and lipopolysaccharide during sepsis may also induce apoptosis [35].

3. Physiopathology of acute respiratory distress syndrome

There are two general types of ALI/ARDS, direct and indirect. Independent of the initial insult, the final result is that alveolar-capillary barrier becomes compromised. Direct ALI/ARDS is often associated with direct mechanical, chemical or infectious stimuli, or other direct interactions capable to induce damage to lung structures [38]. Indirect pulmonary insults such as extra-pulmonary sepsis, trauma, shock, pancreatitis, brain injury or massive transfusion are the mainly causes of indirect ALI/ARDS. However, the highest incidence of indirect ALI/ARDS is seen during sepsis.

The emigration of activated PMNs and passage through the endothelium in the lungs, one of the characteristics of ALI, is regulated via adhesion molecules. Among them, L-selectin (CD62L) on PMNs appears to be involved in the initial rolling proceed on the endothelial surface, while CD11b/CD18 on PMNs mediate a tighter contact between them. CD31 of PECAM-1 is needed in the final step for the vascular diapedesis of leukocytes [38-40]. Neutrophils are able to release a variety of harmful substances, such as proteolytic enzymes, reactive oxygen/nitrogen species, cytokines and chemokynes, which may be injurious to the adjacent endothelial cell and to the alveoli [39]. PMN apoptosis is a crucial injury-limiting mechanism of inflammatory resolution. Several inflammatory agents such as LPS, TNF, IL-8, IL-6, IL-1 and granulocyte colony stimulating factor (G-CSF) can delay apoptotic response, providing PMN with a longer life, allowing accumulating at local tissues [41]. NF κ B has been reported as a modulator of apoptosis in inflammatory cells [42, 43] allowing a pro-inflammatory state.

Loss of epithelial cells and endothelial cell injury are involved in pathogenesis of ALI/ARDS. The former is due to the activation of Fas related apoptosis and the secretion of cytokines and chemokines by lung epithelial cells [44]. The latter is caused by the interaction of endothelial cells with neutrophils that stimulate release of vasoactive compounds, increased pulmonary vascular resistance with pulmonary hypertension [45], but also endothelial cells can be directly stimulated by endotoxin via TLR-1 with the release of vasoactive mediators and molecules altering lung permeability, such as $\text{TNF}\alpha$, thromboxane-A2 and endothelin-1 [46].

Resolution from lung injury is an actively regulated program involving a removal of apoptotic neutrophils, remodeling of matrix, clearance of protein-rich alveolar fluid [47]. Recently, has been demonstrated that CD4^+ lymphocytes as well as plasmacytoid dendritic cells are active players in this process [48, 49].

4. Vectors for gene therapy

Gene therapy is defined as the introduction of nucleic acids into cells for the purpose of altering the course of a medical condition or disease [50]. In general, the advantages of gene therapy over the other treatments are the selective treatment of affected tissues, the possibility of using locally endogenous proteins in cases where its systemic application would incur in serious adverse secondary effects, and the possibility of therapeutic long term after a single application [51]. Currently, there are three categories of gene delivery methods: viral vector based, non-viral vector based and physical methods [52]. Viral-based gene delivery systems is accomplished by using replication-deficient viruses containing the gene of interest, but with the disease-causing sequences deleted from the viral genome [53] including RNA-based viral vectors [54, 55], DNA-based viral vectors such as adenoviral vectors [56], adeno-associated viruses (AAV) vectors or herpes simplex viral vector [57]. The non-viral gene delivery methods use synthetic or natural compounds or physical forces to deliver a piece of DNA into a cell [58]. Two main groups of non-viral delivery methods have developed: chemical-based, including lipofection [59] and inorganic nanoparticles that are usually prepared from metals, inorganic salts or ceramics [60]; and using physical forces such as local or rapid systemic injection [61], particle impact [62, 63], electric pulse [64] or laser irradiation [65].

5. Gene therapy in sepsis

Currently, there is evidence that applying therapeutic maneuvers such as early effective antibiotic administration, intensive fluid resuscitation, mechanical ventilation in selected patients and use of C activated protein in sickest patients improve significantly the survive in these patients [66]. There are several clinical studies that are trying to validate another kind of therapies such as extra-renal depuration, levosimendan, the use of immunoglobulins, nitric oxide, statins, selenium, the use of enteral nutrition with eicosapentaenoic acid (EPA)/ ψ -

linolenic acid (GLA) that are in progress [67]. Basic research and clinical trials have focused on alternative therapeutic approaches [68].

5.1. Pattern associated membrane receptors

Different approaches have designed trying to block the interaction between PAMs and PPRs. One is the generation of antibodies that bind TLRs. Studies conducted with anti-lipopolysaccharide binding protein or anti-CD14 in experimental models of endotoxic shock and Gram-negative bacterial sepsis, failed to show a protection when treatment was administered after LPS or simultaneously with or shortly after bacterial inoculation [69-71]. By using a recombinant chimeric fusion protein composed of the N-terminal and central domains (amino-acids 1-334) of the extracellular part of TLR4 and the Fc portion of the human IgG1, Roger et al [72] produced an anti-TLR4 antibodies that inhibited LPS-induced intracellular signaling and cytokine production and protected mice from lethal endotoxic shock and *E. coli* bacterial sepsis, not only in pre-treatment with the antibodies, but also even when treatment was delayed for several hours after endotoxemia of the onset of sepsis.

The RAGEs (receptor for advanced glycation end products) are part of DAMPs that may play a role in the perpetuation of inflammation that carries to severe sepsis or septic shock. RAGEs are up-regulated in acute and chronic inflammation and bind multiple endogenous mediators involved in sepsis and products of oxidative stress [73]. In a recent work, Christaki et al demonstrated that blocking RAGEs either before or after infection protected mice from lethality in sepsis due to *S. pneumoniae* pneumonia [74] probably by indirect inhibition of NF κ B activation.

Exposure to Staphylococcal enterotoxin (SE) or SE plus lipopolysaccharide (LPS, endotoxin) in mice, triggers vigorous intracellular signaling that leads to hyper-inflammation and release of pro-inflammatory cytokines such as TNF α , INF γ , IL-1 β , IL-1 α , IL-2 and IL-6 by activation of innate immunity [75]. In order to evaluate the role of MyD88, the anchor adaptor protein that integrates and transduces intracellular signals from TLRs and IL-1 receptor superfamily, Kissner et al evaluates a synthetic molecule, hydrocinnamoyl-L-valyl-pyrrolidine (Compd1), which mimics the BB-loop in the TIR domain of MyD88. They observed an inhibited pro-inflammatory cytokine production in human primary cells. Also, administration of Compd1 to mice inhibited pro-inflammatory cytokine response and increased survival from toxic shock induced death-limiting hyper-inflammation [76].

Recently, the knockdown of TLR2 by three different small interfering RNAs (siRNA) (A: 5'-aactatccactgtggaacaa-3', B: 5'-aaactgtgtcagtgccagaaa-3', C: 5'-aaagtcttgattgattggcca-3') reduce de tumorigenesis generated by the injection of BEL-7402 cells in an athymic mouse. Also, the levels of cytokines IL-6 and IL-8 were found to be markedly depressed [77]. In this line, Lei Ming et al have designed four siRNA:

siRNA-180, 5'-GCCUGGAAUACCUUCUAAATT-3';

siRNA-224, 5'-GGGCAGUUCACUGAUUUUATT-3';

siRNA-341, 5'-CAGGAACUGACUCUUGAAATT-3';

siRNA-987, 5'-CCCACUCGGAGAAGUUUAATT-3' against mCD14. *In vitro* experiments with RAW264.7 cells (a transformed murine macrophage cell line) shown that siRNA-224 effectively inhibited LPS-induced TNF α , MIP-2 and IL-6 release and NO production [78].

5.2. Intracellular signaling

Severely burned patients are greatly susceptible to infection with various pathogens [79]. Macrophages (M Φ s) have an important role in antibacterial innate immunity. In methicillin-resistant *Staphylococcus aureus* infection (MRSA), M Φ s (IL-12⁻ IL-10⁻) differentiate in two different subpopulations, M1M Φ (IL-12⁺ IL-10⁻) and M2M Φ (IL-12⁻ IL-10⁺). The former are converted by the TLRs stimulation and has the ability to kill bacteria, to produce reactive nitrogen intermediates, and to release antimicrobial peptides [80], playing a pivotal role in host microbial resistance. M2M Φ have reduced ability to kill bacteria; IL-10 and CCL7 released by M2M Φ are inhibitory molecules on the pathogen-stimulated M Φ conversion to M1M Φ . IL-10 is also a deactivator of antibacterial immunocompetent cells [81] and an inhibitory molecule on various immunocompetent cell functions. Asai et al have demonstrated that IL-10 antisense oligonucleotides in a severely burned mice prevents the burn associated conversion of M Φ to M2M Φ and infectious complications stemming for MRSA local infection did not develop [82].

CCL2 is a chemokine that attracts and activates mononuclear cells. The necessity of this chemokine for Th2-cell generation has been demonstrated. In a study Shigematsu K et al [83] tried to protect thermally injured mice orally infected with a lethal dose of *E. faecalis* by gene therapy utilizing phosphorothioate-CCL2 antisense oligodeoxynucleotides. They demonstrate that sepsis stemming from *E. faecalis* translocation in severely burned mice is controllable by the gene therapy using CCL2 antisense ODNs, through the elimination of mesenteric lymph node macrophages (MLN- M Φ)-M2aM Φ s and M2cM Φ s subtypes. [83].

IL-1 β binds the type-1 IL-1 receptor, while LPS binds to TLR4, both activates intracellular pathways by phosphorylation of IRAK family members including IRAK-1, which involve the MyD88 adaptor protein [84]. The group of Johns RE et al developed a family of "smart" polymeric carriers, termed encrypted polymers that enhance the cytoplasmic delivery of therapeutic antisense oligonucleotides (ASONS). This group has demonstrated that these ASONs block LPS activation of the transcription factor NF κ B reducing the LPS-induced expression of cytokines and chemokines. IL-6 shows a 2-fold decrease whereas TNF α expression trended to decrease. There was a 2-fold decrease in expression of several genes including MCP1, MCP3, eotaxin and IP10 [85].

5.3. Apoptosis

Caspases are pro-enzymes of the aspartate-specific cysteine protease family and its activation plays a central role in the execution of apoptosis [86]. Depending of the stimuli, two caspase-activation pathways have been described, the mitochondria-initiated caspase-8-dependent pathway and mitochondria-initiated caspase-9-mediated pathway. Activation of these pathways initiates a downstream cascade of effector caspases, such as caspase-3 that

cleaves substrates such as D4-GDI leading to cell death [87]. The group of Ayala A et al in 2005 demonstrated that suppression of Fas or caspase-8 gene expression with hydrodynamic administration of siRNA conferred a survival advantage in septic mice model after caecal ligation and perforation (CLP) [88]. In a work of Matsuda N et al, they examined the therapeutic efficacy of caspase-8 and caspase-3 gene silencing with siRNAs delivered by systemic injection in a CLP endotoxic shock mouse model. They demonstrate that *in vivo* delivery of caspase-8/caspase-3 siRNAs conferred a dramatic survival advantage to CLP mice as compared to controls. Also they demonstrated that the survival benefit was observed despite administration of siRNA as late as 10h after CLP [88].

BRCA1 is a critical regulator of DNA damage repair and cell survival. In a recent article, Teoh H et al demonstrated a reduction in 24 hours post caecal ligation and perforation and thioglycollate stimulation mortality with pretreatment with human BRCA1 adenovirus (AdBRCA1). Treatment with AdBRCA1, a human adenovirus type-5 (dE1/E3), blunted CLP-associated cardiac, pulmonary, hepatic and renal dysfunction and also reduced CLP-elicited double strand breaks and apoptosis in the liver. BRCA1 gene therapy was associated with lower CLP-evoked cardiac and hepatic superoxide generation that in the liver was in part due to improved reactive oxygen species removal. CLP also elevated mesenteric arteriolar and serum intercellular adhesion molecule-1, both of which were partially abrogated with AdBRCA1 administration. Thioglycollate-challenged AdBRCA1-treated mice displayed reduced peritoneal neutrophil recruitment and dampened cytokine elaboration relative to their Ad-null-treated counterparts [89].

6. Gene therapy in ARDS/ALI

Over the past 20 years, the feasibility of using gene transfer to treat ALI/ARDS has been demonstrated using a variety of viral and non-viral vectors to deliver various transgenes to the lung [90].

6.1. Strategies to increase pulmonary surfactant

ALI/ARDS is a surfactant-deficient state. *Pseudomonas aeruginosa* infection is a cause of pulmonary infection and ARDS with surfactant deficient phenotype. Zhou J et al have demonstrated the attenuation of the deleterious effects of *Pseudomonas aeruginosa* infection by adenoviral gene transfer overexpressing CCTpenta (a mutant form of the regulatory enzyme CCT α required for the biosynthesis of dipalmitoyl phosphatidylcholine (DPPC), the major phospholipid of surfactant) with a significant increase of the biosynthesis of surfactant. This study suggests that augmentation of DPPC synthesis via gene delivery of CCT α can attenuate impaired lung function in surfactant-deficient states such as bacterial sepsis [91].

6.2. Strategies to improve pulmonary edema

The physiological hallmark of ARDS is disruption of the alveolar-capillary membrane barrier, leading to development of non-cardiogenic pulmonary edema, in which proteinaceous

exudate floods the alveolar spaces, impairs gas exchange and precipitates respiratory failure [92]. Several studies indicate that CLP (cecal ligation and puncture) sepsis model, sepsis and endotoxemia impair the expression of heat shock protein (HSP-70). Data shown that HSP-70 can limit inflammatory responses protect proteins from damage, restore function to proteins that are damaged and prevent cellular destruction, key processes of ALI/ARDS [93]. Weiss et al have demonstrated that the use of an adenoviral vector (AdHSP, an adenovirus carrying the gene for HSP-70) correcting the relative defect in HSP-70 expression prevents neutrophil accumulation, reduce protein rich edema fluid and improve the outcome in ARDS secondary to CLP [94].

Injury of the alveolo-capillary barrier alters active Na^+ transport, leading to impaired edema fluid clearance from the alveolar spaces. Failure to return to normal clearance is associated with poor prognosis [95]. The primary force driving fluid reabsorption from the alveolar space into the interstitium and the pulmonary circulation is active Na^+ transport. Sodium is taken up on the apical surface of the alveolar epithelium by amiloride-sensitive and -insensitive Na^+ channels [96] and is subsequently pumped out of the cell by the Na^+/K^+ -adenosine triphosphatase (Na^+/K^+ -ATPase) on the baso-lateral side [96]. Some studies have demonstrated the importance of Na^+/K^+ -ATPase in ALI/ARDS. In normal adults rats, overexpression of the $\beta 1$ -subunit gene by utilizing a replication-incompetent human type-5 adenovirus expressing Na^+/K^+ -ATPase- $\beta 1$ subunit cDNA increased alveolar edema clearance over two-fold compared with controls [97]. Similarly, gene transfer of the Na^+/K^+ -ATPase- $\beta 1$ subunit using electroporation increased alveolar fluid reabsorption [98]. Furthermore, while rats exposed to 100% oxygen develop ALI and impaired alveolar fluid clearance; overexpression of the Na^+/K^+ -ATPase- $\beta 1$ subunit in the alveolar epithelium of rats increased lung liquid clearance and, most importantly, overexpression of the Na^+/K^+ -ATPase- $\beta 1$ subunit resulted in 100% survival over 14 days of hyperoxia (compared with 25-31% survival in the non-treated or null virus-treated control groups) [99].

In this line, Stern M et al used a cationic liposome to transfer cDNA encoding both α and β subunits of Na^+/K^+ -ATPase to the lung of a mouse model of pulmonary edema induced by thiourea; they observe a significant resolution of pulmonary edema *in vivo*. Also, overexpression of the $\beta 2$ -adrenergic receptor leads to increased alveolar fluid clearance in rats by increasing both membrane-bound amiloride-sensitive Na^+ -channel expression and Na^+/K^+ -ATPase function, probably enhancing responsiveness to endogenous catecholamines in the alveolar epithelium [100].

The regulation of alveolar transport proteins is vital in the maintenance of alveolar fluid balance in patients [101]. The exposure to hypoxia results in decreased Na^+/K^+ -ATPase activity and protein abundance at the plasma membrane by promoting the endocytosis of the pump, which contributes to a decrease in alveolar fluid reabsorption in both *in vivo* and *ex vivo* models of hypoxia. Also, the overexpression of the reactive oxygen species scavenger, SOD2, prevents this hypoxia-mediated decrease in alveolar fluid reabsorption and Na^+/K^+ -ATPase function [102].

6.3. Strategies to afford oxidant injury-related injury, apoptosis and inflammation

Keratinocyte growth factor (KGF) is an epithelial-specific growth factor secreted by fibroblast and vascular smooth muscle cells and a main mitogen for alveolar type II cells [103]. Baba et al have demonstrated that transient over-expression of KGF in the lungs attenuate pathophysiological impairments in hyperoxia-induced acute lung injury by increasing Ki67 and surfactant protein C (Sp-C)-positive cells and proliferation of epithelial cuboidal cells [104]. There is an abundance of evidence regarding the protective effect of pre-treatment with KGF on lung injury induced by hyperoxia, acid instillation, radiation, bleomycin, α -naphthylthiourea, ventilator and bacterial pneumonia there are some studies that supports the potential clinical application of KGF-2 in the treatment of ALI/ARDS [105].

Human angiopoietin-1 (ANGPT1), a ligand for the endothelial-restricted receptor TEK tyrosine kinase, plays an essential role in blood vessel maturation and stabilization during embryonic development. In postnatal, ANGPT1 maintains the normal quiescent phenotype of vascular ECs, protecting against vascular inflammation reducing permeability and promoting ECs survival. In a study of Mei SH and co-workers carried out in an ALI mice model (by intra-tracheal instillation of LPS), they have demonstrated that mesenchymal stem cells (MSCs) administration alone into the pulmonary circulation partially prevents LPS-induced lung inflammation. However, cell-based gene transfer using pANGPT1-transfected MSCs resulted in further improvement in both alveolar inflammation and membrane permeability. Also, MSCs-pANGPT1 dramatically reduced cytokine levels ($\text{IFN}\gamma$, $\text{TNF}\alpha$, IL-6 and IL-1 β) to the baseline values observed in naïve mice, suggesting a potential therapeutic approach to ALI/ARDS [106].

Pearl M and colleagues in a 2005 study using Fas- and caspase-8 siRNA intra-tracheal administration in a CLP mice model of sepsis demonstrated that the main targets of siRNA delivery are the epithelial cells. Also, that down-regulation of Fas but not caspase-8 reduces pulmonary apoptosis and lung inflammation, decreases neutrophil influx and attenuates ALI [107].

Overexpression of interleukin IL-10 through recombinant adeno-associated virus type-5 (AAV5) vector expressing murine IL-10 into pulmonary, tissue proinflammatory cytokines IL-1 β and $\text{TNF}\alpha$, macrophage inhibitory protein-1 α and keratinocyte chemoattractant in the epithelial lining fluid and lung homogenate were decreased and neutrophil infiltration was less pronounced and more localized neutrophil infiltration in lung section [108].

Finally, Hemoxygenase-1 (HO-1) is an inducible isoform of the first and rate-controlling enzyme of the degradation of heme into iron, carbon monoxide, and biliverdin, the latter being subsequently converted into bilirubin. Several positive biological effects exerted by this enzyme have gained attention, as anti-inflammatory, antiapoptotic, angiogenic, and cytoprotective functions are attributable to carbon monoxide and/or bilirubin. Also, the enzyme has been involved in controlling infiltration of neutrophils into the injured lung and in the resolution of inflammation by modulating apoptotic cell death and cytokine expression. Several groups have delivered HO-1 expressing adenoviruses to the lungs in both pneumonia and

hyperoxia models and have shown significant reductions in inflammation and subsequent lung injury [90].

7. Future directions and conclusion

Sepsis and acute lung injury/acute respiratory distress syndrome are important pathologies in critical care medicine. There are increasing evidence from relevant pre-clinical studies that support the efficacy of gene-based therapies. Multiple barriers exist to the successful use of gene therapy in critical care medicine and particularly in sepsis and ALI/ARDS. Future research approaches are necessary to overcome these barriers by developing better viral and non-viral vectors, enhanced and specific gene expression strategies, improved cellular uptake of vectors and better therapeutic targets.

Although the treatment by transference of genetic material still presents many challenges, the technology is rapidly evolving and the possible use in clinical trials could be in a near future. So, the aim of this chapter was to understand the molecular mechanisms involved in acute respiratory distress syndrome and sepsis, to review the viral and non-viral gene therapies that have been developed to improve survival and to address the challenges of gene therapy in critical care patients using these two life-threatening conditions as a model.

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Clinical and Translational Challenges in Gene Therapy of Cardiovascular Diseases

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1. Introduction

Cardiovascular (CV) disease is the most prevalent life-threatening clinical problem and is a major cause of disability and economic burden worldwide [1]. Despite extensive pharmacotherapies, there remain many vascular conditions for which pharmacological interventions are either non-existent or largely ineffective. CV gene therapy offers the benefit of sustained and/or controlled expression of desired proteins in cell types, which makes it more beneficial in providing durable clinical benefits [2]. The therapeutic gene works by either over-expressing therapeutically beneficial proteins, replacing a deficient gene or its expression proteins, or silencing a particular gene whose expression is not beneficial in the clinical scenario [3]. In addition, success of gene therapy also depends on the choice of the vector and the delivery approach. Blood vessels are among the most feasible targets for gene therapy because of ease of access using a catheter or by systemic delivery. The new genetic material should enter the cells in the vasculature overcoming the anatomical, cellular and physiological barriers and induce the expression of the transfected gene in the target tissue. The target cells in the arteries are endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts, which constitute the intimal, medial and adventitial layers, respectively [4]. In the case of atherosclerotic lesions, macrophages also become a target cell. For the treatment of cardiovascular diseases, gene therapy strategies have been designed to enhance re-endothelialization and EC function to reduce thrombosis, inhibit SMC proliferation and migration to prevent neointimal hyperplasia, and to improve therapeutic neo-vascularization to counteract ischemia.

Viral and non-viral vector systems have been evaluated for gene transfer to the vasculature. Lipoplexes, polyplexes and lipopolyplexes as well as naked DNA have been used as non-viral vectors for gene delivery to vascular tissues. Retroviruses, lentiviruses, adenoviruses

and adeno-associated viruses have been tested as viral vectors. Both systems have their own advantages and disadvantages that determine its use for a particular subset of CV diseases. Another challenge is the development of delivery approaches that are clinically viable and are capable of achieving consistent therapy for diseased arterial tissues. The efficiency of localization, restriction of systemic distribution and adequacy of permeation into the target tissue are required for the optimal delivery of the vector. It is also dependent on the requirements of a given patho-physiological situation. Systemic, intravascular and perivascular approaches are used for gene delivery to the vasculature.

In this chapter, our goal is to summarize the current understanding of gene therapy strategies used to treat CV diseases, specifically the therapies targeting thrombosis, atherogenesis, SMC proliferation and migration, modification of extracellular matrix (ECM) and regeneration of the endothelial cell layer. We will discuss various vectors and delivery approaches used in the CV gene therapy and describe, in detail, the challenges associated with each approach.

2. Vectors in vascular gene therapy

The ideal vector for clinical application would target the specific cell, offer the capacity to transfer large DNA sequences, result in therapeutic levels of transgene expression that are not attenuated by the host immune response, express transgene for a duration required to alleviate the clinical problem, pose no risk of toxicity either acutely (as a result of immunogenicity or unregulated transgene expression) or in the long-term (such as oncogenesis), and be cost-effective and easy to produce in therapeutically applicable quantity [5]. Currently, no available vector fulfils all these criteria; therefore, a perfect vector for vascular gene therapy does not exist. Nonetheless, viral and non-viral vector systems have been evaluated for gene transfer to the vasculature.

2.1. Viral vectors

Retroviruses, adenoviruses (Ad) and adeno-associated viruses (AAV) are used as viral vectors in vascular gene transfer. Recombinant retroviruses are RNA viruses that are capable of integrating transgene into the target genome. Disadvantages of this vector include instability, the requirement of cell division for gene transfer and the inability to attain high titers. Since the majority of vascular cells are not undergoing mitosis at the time of exposure to the viral vector, the efficiency of gene delivery to vascular cells by such vectors may be as low as 1% to 2% [6]. Attempts have been made to increase the transduction efficiency in endothelial cell using multiple viral exposures [7] or increasing viral titers by ultracentrifugation [8]. Murine leukemia retroviral vectors (MuLV) pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) have the capacity to transfect human ECs and SMCs *in vitro* with significant improvement in stability and transduction efficiency [9]. Unlike other retroviruses, lentiviruses are able to transduce non-dividing cells, which is an attractive characteristic for CV gene therapy. These vec-

tors demonstrate significantly broadened tropism and high stability and have been used to demonstrate efficient transgene delivery *in vitro* into SMCs and ECs from human saphenous vein [10], human coronary artery SMCs and ECs [11], and cardiomyocytes [12].

Ad vectors are the most commonly used viral vectors in the CV system. They transfect non-dividing cells efficiently [Figure 1], but sustained gene expression is limited to approximately 2 weeks because the gene is kept episomal [2]. The administration of the Ad vectors is almost invariably associated with the development of systemic neutralizing antibodies directed against the vector [13]. Therefore, lowering the immunogenicity of the Ad virus is desirable and can be achieved by deleting genes that encode viral proteins [14]. Another method of reducing the inflammatory reaction to gene transfer by Ad vectors is to preserve the E3 region, which is supposed to modulate the host immune response *in vivo* [15]. When systemically administered, Ad5 poorly transduced ECs but could effectively transduce medial SMCs during endothelial denudation [5]. Efficient myocardial transduction was observed following local delivery of Ad5 vectors in porcine heart, where almost 80% of cardiomyocytes were transduced [16].

AAV vectors have emerged as versatile vehicles for gene delivery due to their efficient infection of dividing and non-dividing cells in the presence of helper virus, sustained maintenance of viral genome leading to long-term expression of the transgene, and a strong clinical safety profile [17]. AAV is non-pathogenic since it cannot replicate without the assistance of a helper virus. Recombinant AAV (rAAV) vectors have almost the entire viral genome removed, thereby yielding a delivery vehicle with enhanced safety and reduced immunogenicity [18]. The AAV *Rep* and *Cap* genes, which are required for viral replication and packaging, are supplied by a helper plasmid during the production process. Wild type AAV preferentially integrates to a specific locus of human chromosome 19. The rAAV has mechanisms for sustained episomal maintenance or semi-randomly integrates at a low rate [19]. Problems with AAV vectors include limited tissue tropism for serotypes that bind heparan sulphate, challenges with preexisting immunity due to prior exposure, and also substantially delayed onset of transgene expression compared to other vectors.

2.2. Non-viral vectors

Even though the transfection efficiency of non-viral vectors are lower than that of their viral counterparts, they are associated with many advantages such as low immunogenic response, the capacity to carry large inserts of DNA (52Kb), the possibility of selective modification using ligand and large scale manufacture [20]. Ideal non-viral vectors should be degradable into low molecular weight components in response to biological stimuli for lower toxicity and effective systemic clearance. They should also be efficient in overcoming extracellular and intracellular barriers and tissue/cell-targeted for specific accumulations [21]. In this group of vectors, naked DNA, cationic liposomes and cationic polymers have been used for vascular gene transfer.

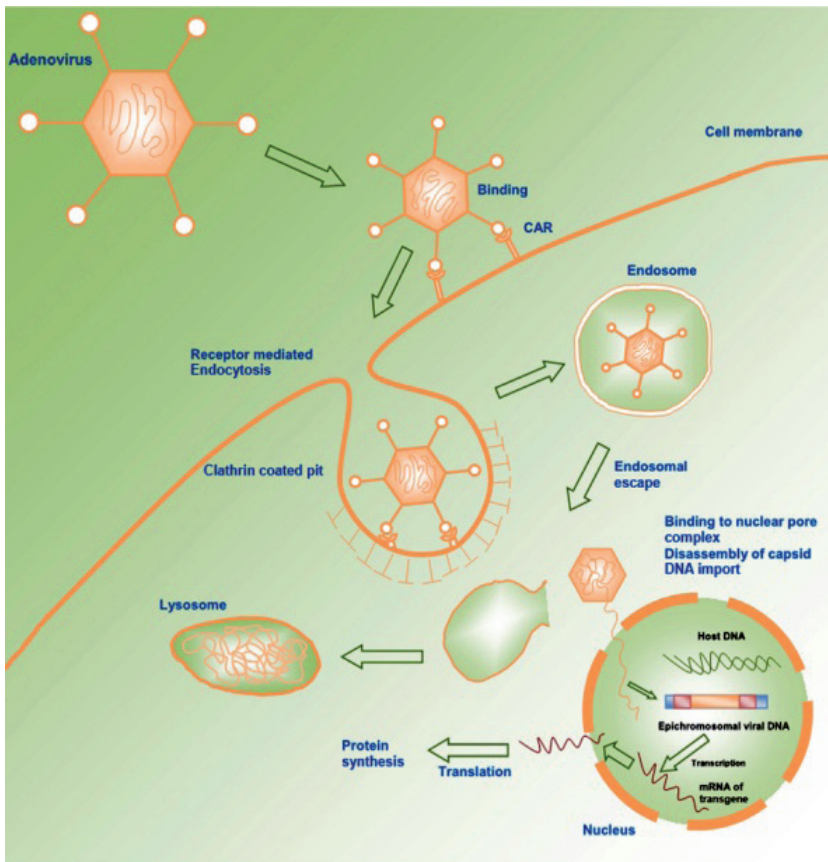


Figure 1. Transduction using adenoviral vectors. Recombinant adenovirus enters cells via CAR-mediated binding allowing internalization via receptor-mediated endocytosis through clathrin-coated vesicles. Inside the cytoplasm, the endocytosed adenoviral vector escapes from the endosomes, disassembles the capsid and the viral DNA enter into the nucleus through the nuclear envelope pore complex. The viral DNA is not incorporated into the host cell genome, but rather assumes an epichromosomal location, where it can still use the transcriptional and translational machinery of the host cell to synthesize recombinant protein. [CAR; Coxsackievirus and adenovirus receptor]

Gene transfer with naked DNA is attractive because of its simplicity and lack of toxicity [22]. However, the efficiency of gene transfer with naked DNA is low due to its negative charge conferred by the phosphate groups, making cellular uptake difficult by the negatively charged cell surface, rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system in the systemic circulation. However, site-specific arterial gene transfer of vascular endothelial growth factor (VEGF)-165 could yield efficient gene transfection resulting in accelerated re-endothelialization, inhibition of neointimal

thickening, reduced thrombogenicity, and restoration of endothelium-dependent vasomotor reactivity after injury due to balloon angioplasty in a rabbit model [23]. Physical approaches have been explored for plasmid gene transfer into vascular cells *in vitro* and *in vivo*. Ultrasound exposure can induce transient pore formation in the cell membrane, thereby increasing the plasmid DNA uptake. Indeed, microbubble-enhanced ultrasound can achieve transgene expression levels *in vitro* at approximately 300-fold than that of naked plasmid DNA alone in porcine VSMCs [24]. The non-invasive nature of this technique makes it more feasible for clinical use. Local administration of plasmid DNA, coupled with application of brief electric pulses to cells or tissues to increase cellular permeability-- also called electroporation--yields high levels of transgene expression in the arteries [25]. However this technique is limited by its invasive nature and tissue damage associated with high voltages applied [26].

To increase the efficiency of gene transfer by naked DNA, they are complexed with cationic lipids (liposomes or lipoplexes) or polymers (polyplexes). The resulting net positive charge of the cationic lipid/polymer DNA complexes facilitates fusion with the negatively charged cell membrane and also reduces susceptibility to circulating nucleases. Transfection efficiency of cationic lipoplexes varies dramatically depending on the structure of the cationic lipids (the overall geometric shape, the number of charged groups per molecules, the nature of lipid anchors, and linker bonds), the charge ratio used to form DNA-lipid complexes, and the properties of the co-lipid [22]. Although transfection efficiencies of liposomes are generally seen lower in vascular cells [22], the LID vector system, consisting of a liposome (L), an integrin targeting peptide (I), and plasmid DNA (D), transfects primary porcine vascular SMCs and porcine aortic ECs with efficiency levels of 40% and 35%, respectively, under *in vitro* conditions [27]. Some of the cationic lipids have been found to negatively affect cell function. Cationic lipid-mediated transfection of bovine aortic ECs inhibits their attachment [28].

The DNA packaging efficiency and *in vivo* stability are higher for cationic polymers compared to cationic lipids. Furthermore, these complexes can be surface-modified with antibodies or other targeting ligands to deliver nucleic acids to specific cells [29]. Several cationic polymers have been evaluated for their ability to form complexes with DNA, the most significant being poly-lysine (PLL) and polyethylene-imine (PEI) [30]. PEI affects EC function [31]; however, when conjugated with fractured polyamidoamine (PAMAM) dendrimers, less toxic effects were observed on vascular cells in addition to the enhanced transfection efficiencies [32]. Brito *et al.* [33] developed lipo-polyplex nanovector systems that can transfect EC and SMCs with reasonably high efficiency. They used a combination of a cationic biodegradable polymer, poly(beta-amino ester) (PBAE), and a cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and obtained 20% and 33% transfection efficiencies *in vitro* in SMC and ECs, respectively. Molecular tuning of non-viral vectors via stimuli responsive degradation is another novel approach that can be adopted in vascular gene transfer [21]. Schematic representation of non-viral gene delivery is given in Figure 2.

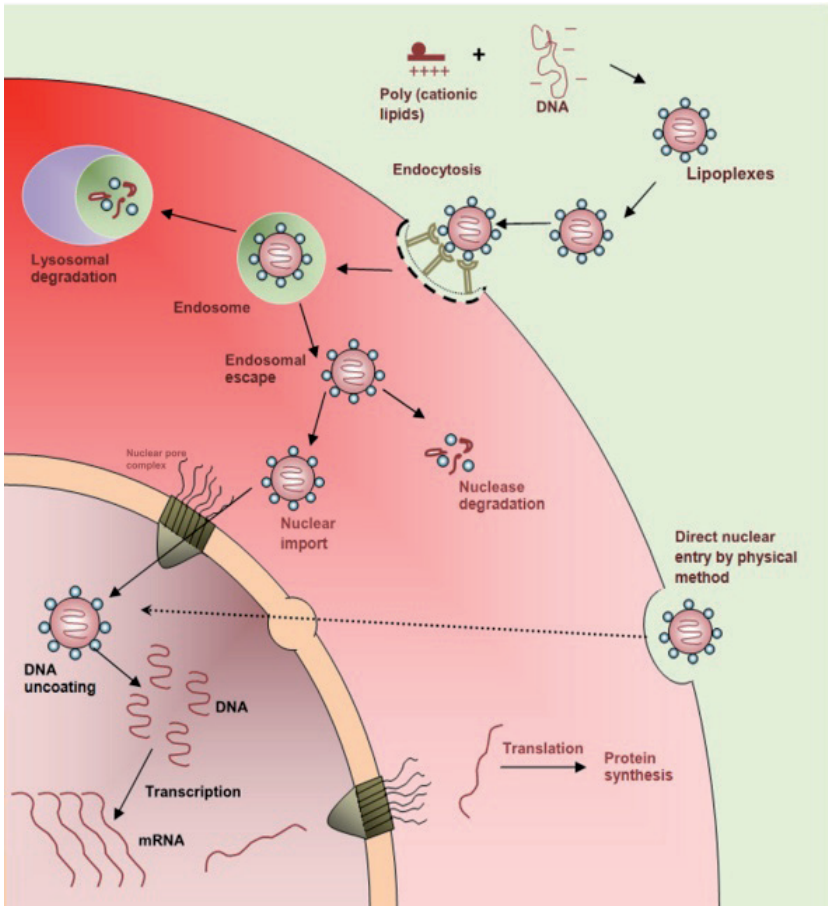


Figure 2. Non-viral gene delivery using lipoplexes: DNA is complexed with cationic liposomes and is internalized through receptor mediated endocytosis. After their internalization large amounts of complexes are degraded in the endolysosomal compartments. Only a small fraction enters into the nucleus and elicits desired gene expression.

2.3. Stem cells

One of the recent approaches is to use stem cells as gene delivery vehicles. Stem cell-based gene therapy approaches are currently being employed in recent studies as an alternative strategy to promote myocardial angiogenesis and regeneration. Indeed, the injection of genetically modified bone marrow-derived mesenchymal stem cells to express angiopoietin-1 improved arteriogenesis and increased collateral blood flow in porcine model of chronic myocardial ischemia [34]. Nanofiber-expanded hematopoietic stem cells over-expressing

VEGF and platelet-derived growth factor (PDGF) had a favorable impact on the improvement of rat myocardial function accompanied by upregulation of tissue connexin 43 and pro-angiogenic molecules after infarction [35].

3. Major targets in vascular gene therapy

3.1. Promotion of re-endothelialization

EC loss because of vascular injury is a major contributing factor to the local activation of patho-physiological events leading to the development of neo-intimal hyperplasia [36]. Previous reports have shown that transplantation of autologous endothelial progenitor cells (EPCs) onto balloon-injured carotid artery leads to rapid re-endothelialization of the denuded vessels [37]. EPCs can be genetically manipulated *ex vivo*, expanded, and reintroduced *in vivo*, where at least a proportion will contribute to a long-lasting pool that can provide therapeutically relevant levels of transgene expression. Chemokine receptor, CXCR4, is a key molecule in regulating EPC homing [38]. Chen *et al.* [38] reported that CXCR4 gene transfer to EPCs contributes to their enhanced *in vivo* re-endothelialization capacity. In another study, Ohno and colleagues over-expressed C-type natriuretic peptide by gene transfer in rabbit jugular vein grafts and observed accelerated re-endothelialization [39]. EPCs over-expressing endothelial nitric oxide synthase (eNOS) further enhance the vasculo-protective properties of these cells [40]. Local intravascular and extra-vascular expression of VEGF, using plasmid DNA, accelerated re-endothelialization and decreased intimal thickening after arterial injury in rabbit models [23, 41].

3.2. Promotion of endothelial cell function

Antithrombotic and anticoagulation therapy generally involves the systemic administration of agents that target a small region of the vasculature. Localized and controlled delivery of specific genes could allow sustained antithrombotic or anticoagulant treatment when prolonged systemic administration is undesirable. Antithrombotic gene therapy strategies could include inhibition of coagulation factors, over-expression of anticoagulant factors, or modulation of EC biology to make thrombus formation or propagation unfavorable [42]. Ad gene transfer of thrombomodulin decreased arterial thrombosis to 28% compared to 86% in control rabbit model [43]. Hemagglutinating virus of Japan (HVJ)-liposome-mediated gene transfer of tissue factor pathway inhibitor (TFPI), a primary inhibitor of TF-induced coagulation, significantly reduced/inhibited thrombosis after angioplasty in atherosclerotic arteries without any significant adverse effects [44]. Ad gene transfer of many mediators, including hirudin to inhibit thrombin [45], tissue plasminogen activator (tPA) to enhance fibrinolysis [43], cyclo-oxygenase to augment prostacyclin synthesis [46], prevents arterial thrombosis and promotes local thromboresistance. Vascular gene delivery of anticoagulants by local infusion of retrovirally-transduced EPCs with tPA and hirudin genes has also been attempted [37].

3.3. Inhibition of atherogenesis

The extensive cross-talk between the immune system and vasculature leading to the infiltration of immune cells into the vascular wall is a major step in atherogenesis. In this process, reactive oxygen species play a crucial role, by inducing the oxidation of low-density lipoprotein (LDL) and the formation of foam cells, and by activating a number of redox-sensitive transcriptional factors, such as nuclear factor kappa B (NF κ B), Nuclear factor E2-related factor-2 (Nrf2) [47], or activating protein 1 (AP1) that regulate the expression of multiple pro- and anti-inflammatory genes involved in atherogenesis [48]. Delivery of genes encoding antioxidant defense enzymes, like extracellular superoxide dismutase [49, 50], catalase [51], glutathione peroxidase [51] or heme oxygenase-1 [52], suppresses atherogenesis in animal models.

Apolipoprotein E (ApoE), a blood circulating protein with pleiotropic atheroprotective properties, has emerged as a strong candidate for treating hypercholesterolemia and CV disease. The gene transfer of ApoE Ad vectors produced substantial amounts of plasma ApoE following intravenous injection into ApoE $^{-/-}$ mice, which lowered plasma cholesterol, and after 1 month, slowed aortic atherogenesis [53]. Hepatic expression of human ApoE3 using a second-generation recombinant Ad vector directly induced regression of pre-existing atherosclerotic lesions without reducing plasma cholesterol or altering lipoprotein distribution [54]. High concentrations of atherogenic apolipoprotein (apo) B100 could also be lowered by hepatic gene transfer with the catalytic subunit of apoB mRNA editing enzyme [55].

3.4. Inhibition of SMC proliferation and migration

SMC migration and proliferation as well as deposition and turnover of ECM proteins contribute to the process of intimal hyperplasia. Several different approaches were introduced to inhibit SMC proliferation during restenosis. Most of the approaches targeted inhibition of cell cycle, where cell cycle inhibitor genes are over-expressed. Non-phosphorylated retinoblastoma gene (Rb) [56]; p21 [57, 58]; p27-p16 fusion gene [59, 60]; cyclin-dependent kinase inhibitor p57Kip2 [61]; and the growth-arrest homeobox gene *gax* [62] are few of the genes over-expressed to inhibit cell proliferation and neo-intimal formation. Genes that have a beneficial influence on various aspects of vessel wall physiology also inhibit SMC proliferation. Nitric oxide generation by endothelial nitric oxide synthase inhibits SMC proliferation *in vitro* and modulates vascular tone locally *in vivo* [63].

Another approach was to inhibit growth factor signaling by the introduction of nucleic acid constructs that interfere with mRNA stability, such as antisense oligonucleotides, hammerhead ribozymes and siRNA [64]. Gene transfer of a truncated form of fibroblast growth factor (FGF) receptor using Ad vector suppressed SMC proliferation *in vitro* [65]. Hammerhead ribozymes directed against PDGF-A chain [66] and transforming growth factor- β [67] inhibited SMC proliferation and neointima formation in rat carotid artery after balloon injury.

The regulation of a target gene can influence the level of transcription, either by decoy oligonucleotides, which are either short double-stranded oligonucleotides or dumb-bell shaped circular oligonucleotides that represent transcription factor binding sites, and thus compete

for binding of a specific transcription factor that is relevant for the respective gene [64]. Administration of AP-1 decoy ODNs *in vivo* using HVJ-liposome method virtually abolished neointimal formation after balloon injury to the rat carotid artery [68]. Transfection of vein grafts with a decoy antisense oligonucleotide to block transcription factor E2F imparted long-term resistance to neointimal hyperplasia and atherosclerosis in rabbits on a cholesterol diet [69]. Another approach was to drive SMC into apoptosis during the process of proliferation and migration. Transduction of rabbit iliac arteries with recombinant Ad vectors for Fas ligand (L) reduced neointima formation, which occurred through the killing of Fas expressing neighboring SMC by FasL-transduced cells [70].

The regulation of SMC migration is mediated partly through the action of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) [71]. AAV-mediated TIMP1 transduction in SMCs of injured rat carotid arteries significantly reduced the ratio of intima to media (52.4%) after two months of treatment [72]. Overexpression of TIMP-2 [73], TIMP-3 [74] and TIMP-4 [75] has also been demonstrated to inhibit SMC migration and neo-intimal proliferation in human vein grafts and porcine vascular injury models. Gurjar *et al.* [76] demonstrated that eNOS gene transfer inhibits SMC migration and MMP-2 and MMP-9 activities in SMCs *in vitro*. A combination approach of TIMP-1 and plasminogen activator system inhibited vein graft thickening in hypercholesterolemic mice, when plasmids encoding TIMP-1-ATF (amino terminal fragment of urokinase) were incorporated to the vein graft by intravascular electroporation [77].

3.5. Enhancement of therapeutic angiogenesis

Ischemic diseases, including acute myocardial infarction and chronic cardiac ischemia, are characterized by an impaired supply of blood resulting from narrowed or blocked arteries that starve tissues of needed nutrients and oxygen [78]. Delivery of genes encoding angiogenic factors or the whole protein has been shown to induce angiogenesis in numerous animal models with the expression of a functioning product [79]. The successful application of recombinant protein and gene transfer for the treatment of myocardial ischemia was reported by Losordo and colleagues [80] by direct intra-myocardial gene transfer of naked plasmid DNA encoding VEGF-165 in porcine model. These results were confirmed in phase 1 assessment of direct intra-myocardial administration of Ad vector expressing VEGF-121 cDNA in patients with severe coronary artery disease [81]. Ad-mediated FGF-4 gene transfer improved cardiac contractile function and regional blood flow in the ischemic region during stress in pig model [82]. Placebo-controlled trials in humans with chronic stable angina indicate that Ad5FGF-4 increased treadmill exercise duration and improved stress-related ischemia [82]. In another study, following coronary artery occlusion, rabbits treated with Ad vector containing acidic FGF showed a 50% reduction in the risk region for myocardial infarction [83].

4. Challenges in gene therapy

4.1. Cellular and extracellular barriers in gene delivery

Viruses have highly evolved mechanisms for obtaining optimized receptor-mediated internalization, efficient cytosolic release, directed and fast intracellular transport towards compartments and readily disassemble. In contrast, non-viral vectors must overcome multiple extracellular and intracellular barriers [21]. These barriers include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelope. To overcome the delivery barriers in non-viral gene transfer, various strategies have been employed to enhance the circulation time, improve intracellular delivery, and enhance endosomal escape and nuclear import. Lipoplexes have shown rapid hepatic clearance during systemic administration. Modification of lipoplexes with hydrophilic molecules like polyethylene glycol (PEG) and polyethyleneimine (PEI) causes steric hindrance between opsonins and the delivery vectors, increasing their circulation time in the blood. PEGylation of PLL decreases interparticle aggregation, resulting in high transfection efficiency in the presence of serum [29]. One study has demonstrated that when artery wall binding peptide (AWBP), a core peptide of apo B100 -- a major protein component of LDL -- was conjugated to PLL with PEG as the linker, the PLL-PEG-AWBP protected the plasmid DNA from nucleases for more than 120 min in circulation and also showed 100 times higher transfection efficiency when compared to PLL and PLL-g-PEG in bovine aortic ECs and SMCs [84]. In an innovative approach, micellar nanovectors made of PEG-block-polycation, carrying ethylenediamine units in the side chain [PEG-PAsp(DET)], complexed with plasmid DNA to form polyplex micelle effectively transfected vascular smooth muscle cells in vascular lesions without any vessel occlusion by thrombus [85] in rabbit carotid arteries. However, PEI-mediated gene delivery can affect EC function and viability [31].

The size and charge of the lipoplex/polyplex play an important role in their intracellular delivery. Lipoplexes and polyplexes are generally formulated into particles with net positive charges to trigger endocytosis by non-specific electrostatic interaction between the positively charged complexes and negatively charged cell surface [29]. Since drug carriers with a smaller particle size have resulted in higher arterial uptake compared to carriers with larger size, the size of the complexes was expected to be a dominating factor in the arterial wall lesions because of the rapid blood flow which could wash out most of the drugs or therapeutic chemical agents from the arterial wall lesions within 20–30 min. Song *et al.* [86] reported a potentially useful particle size of 70~160 nm for local intraluminal therapy of restenosis.

By taking advantage of high expression levels of receptors or antigens in diseased conditions, gene complexes can be targeted using specific ligands, such as antibodies, peptides and proteins. Cyclic RGD (cRGD) peptide recognizes $\alpha(v)\beta(3)$ and $\alpha(v)\beta(5)$ integrins, which are abundantly expressed in vascular lesions. When cRGD was conjugated to PEG-PAsp(DET) to form polyplex micelles through complexing with plasmid DNA, the micelles achieved significantly more efficient gene expression and cellular uptake as compared to PEG-PAsp(DET) micelles in ECs and SMCs [87]. PAMAM dendrimers with E/P-selectin an-

tibody was used for gene targeting to activated vascular ECs [88]. The lectin-like oxidized LDL receptor (LOX-1) is expressed selectively at low levels on ECs but is strongly upregulated in dysfunctional ECs associated with hypertension and atherogenesis. White and colleagues [89] confirmed the selectivity to LOX-1 for peptides LSIPPKA, FQTPPQL, and LTPATAI, which could be potential targets to dysfunctional ECs expressing LOX-1 receptor. Another approach to increase intracellular delivery is to use cell penetrating peptides (CPPs). CPPs consist of short peptide sequences that are able to translocate large molecules into the cells and increase the transfection efficiency [90].

Following internalization of lipoplexes and polyplexes via endocytosis, endosomal entrapment and subsequent lysosomal degradation are the major hurdles that limit transfection efficiency [29]. Lipoplexes are modified with dioleoylphosphatidylethanolamine (DOPE) or other helper lipids due to its fusogenic functionality and its ability to destabilize endosomal membranes. Small PLLs with cationic lipid DOCSPEER [1,3-dioleoyloxy-2-(N(5)-carbamoylspermine)-propane] enhanced gene transfer in primary porcine SMCs *in vitro* and *in vivo* in porcine femoral arteries [91]. Polyplexes, PEI and PAMAM are cationic polymers of high efficiency partly because of their ability to burst the endosomal membrane due to 'proton sponge effect'.

A promising new delivery strategy is to use synthetic peptide carriers containing a nuclear localization signal to facilitate nuclear uptake of plasmid DNA. Nuclear import of plasmid DNA is more challenging for transfecting non-dividing cells. Strategies to increase the nuclear import of genes involve tagging the nuclear localization sequence (NLS) with DNA vectors. NLS is a major player that shuttles protein-plasmid complexes through the nuclear pore by interaction with importins and transportin [92, 93]. Incorporation of DNA nuclear targeting sequence SV40 into expression plasmids results in 10-40 fold increases in vascular gene expression in rat mesenteric arteries [94], confirming the function of DNA nuclear targeting sequences *in vivo*.

4.2. Challenges associated with the vectors

4.2.1. Insertional mutagenesis

Insertional mutagenesis is a major concern in gene therapy involving viral vectors. These vectors integrate randomly or quasi-randomly into the host cell's genome, to stably transfect the target cell. The variable site and frequency of integration of the transgene can induce mutagenesis in the host genome, resulting in devastating consequences for the cell and for the organism. [95, 96]. Another disadvantage of the random integration of a transgene is the unpredictability of its stability and its expression. The genomic locus in which the vector integrates can have profound effects on the level of transgene expression, as it can completely silence the transgene, or it can increase or decrease its expression. These effects could not be avoided by sophisticated vector design or inclusion of the gene's own promoter and/or enhancer region in the transgenic vector construct, as the surrounding chromatin can override the activity of the original regulatory regions. Gene targeting by homologous recombination, however, lacks many of these shortcomings [96]. In this process, the transgene recombines

with its natural locus in the host genome, thereby ensuring correct transcription. Also, after homologous recombination, the targeted modification of the chromosomal locus is stable, whereas randomly integrated sequences might be lost over time. In their seminal paper, Russel and Hirata [97] reported that DNA vectors based on the AAV could target homologous chromosomal DNA sequences and allow high-fidelity, non-mutagenic gene repair in a host cell. Although the laborious vector design and low transfection efficiencies of AAV vectors compared to the other viral vectors still remains a concern, statistical information neatly outlines the advantage of rAAV gene replacement system over standard viral vectors, which induce strong immune response.

4.2.2. Tissue-specific targeting

The promiscuous tropism of vectors resulting in high-level transgene expression in multiple tissues is another major challenge in vascular gene therapy. After systemic application, most viral vectors are trapped by the liver, hampering delivery to target CV tissues. Approaches to restrict gene delivery to desired cell types *in vivo* relied mostly on cell surface targeting or cell-specific promoters.

The *cis*-acting regulatory elements of the SM (smooth muscle)22 α [98-100], telokin [101], smooth muscle myosin heavy chain [102], smooth muscle α - [100] and γ -actin [103], and desmin [104] genes have been shown to direct reporter gene expression to smooth muscle tissues in transgenic mice. In our studies, specific gene transfer to the SMC layer was achieved in swine coronary and peripheral arteries using SM22 α promoter in AAV [17]. Although the efficiency of transduction was low when compared to a similar study using AAV vectors with cytomegalovirus (CMV) promoter [105], the use of SM22 α promoter caused specific transduction of SMCs *in vivo*. An interesting approach to enhance the transduction efficiency of SM22 α -containing plasmid was to incorporate chimeric transcriptional cassettes containing a SM-myosin heavy chain enhancer element combined with the SM22 α promoter [106]. The transfection levels obtained using these chimeric constructs in Ad vector were similar to that with CMV promoter when tested in rat carotid arteries. Certain DNA nuclear targeting sequences can be used to restrict DNA nuclear import to specific cell types. Young *et al.* [107] improved the efficiency of transduction in SMCs of rat vasculature using a SMC-specific DNA nuclear targeting sequence.

EC specific gene expression was obtained when promoters of *fms*-like tyrosine kinase-1 (FLT-1) [108], intercellular adhesion molecule (ICAM) -2 [109], angiopoietin-2 [110], eNOS [111], vascular cell adhesion molecule-1 (VCAM-1) [112], von Willebrand factor [113], tyrosine kinase with immunoglobulin and epidermal growth factor homology domains (Tie) [114], kinase-like domain receptor [115] were used in transgenic mouse models. Other EC-specific promoters include the oxidized LDL receptor LOX-1 [116] and ICAM-1 [117], which exhibit upregulation upon cytokine stimulation, a possible advantage depending on the application in inflammatory conditions [118]. With the possible exception of the mouse Tie-2 and human ICAM-2 genes, most of EC-specific promoters tested to-date have been shown to direct expression in distinct and restricted sites of the vascular tree [119]. A combination

approach of the Tie2 promoter and enhancer (Tshort) by Minami and colleagues [119] directed widespread EC expression *in vivo*.

Another challenge was in generating an EC-specific promoter with comparable efficiency as the CMV promoter. White *et al.* [120] examined several novel Ad expression cassettes for EC-specific gene transfer with CMV, Tshort, ICAM-2, ICAM-1, FLT-1 promoters, respectively and found that LOX-1 promoter elements significantly increased reporter gene expression in carotid arteries compared to other promoters. The efficacy of these novel expression cassettes in large animal models have yet to be established.

An increasingly important area to in-tissue specific targeting is to engineer viral vectors Ads and AAVs with altered cell tropisms to narrow or broaden its efficiency in tissues refractory to infection [19, 121]. Non-genetic approaches typically utilize bispecific antibodies that both neutralize wild-type virus tropism and provide a new cell binding capacity [122]. For genetic targeting strategies, the virus capsid are engineered to express foreign ligands that target selected receptors in the absence or presence of additional modification to ablate the natural tropism of the virus [122, 123]. Ad homing to target endothelial cells at specific sites of the body can be achieved by deleting the ability of the virus to interact with its natural receptor, Coxsackievirus and adenovirus receptor (CAR), and a simultaneous addition of a ligand that directs the virus to the angiotensin converting enzyme on the ECs. Retargeting of AAV-2 with novel peptides could increase both transduction efficiency and selectivity [124] in vascular ECs [125] and SMCs [126] *in vitro*.

4.3. Challenges associated with the mode and route of gene delivery

4.3.1. Systemic gene delivery

The vascular system represents an ideal route of substance transport for reaching a specific site for therapeutic intervention. However, in the case of non-viral vectors, which are cationic polymers in most cases, it has been found that electrostatic interactions between the sulphated glycosaminoglycans in the serum as well as those expressed on the cell surface cause premature release of plasmid DNA leading to its inactivation and extracellular degradation by serum DNases [21]. Also, after systemic vascular application, non-specific distribution of plasmid DNA throughout the vasculature would result in undesired side effects because of accumulation at non-specific sites. Intravenous administration of cationic polymers resulted in their localization to liver, lung, kidney, and spleen in pigs and rabbits [127-129]. Other barriers to systemic delivery include rapid clearance of the lipoplexes by the reticulo-endothelial system and target specificity.

Most Ad vectors are trapped by the liver, hampering delivery to target CV tissues after systemic application. Systemic tail vein injection of Ad vector in mice resulted in virus DNA deposition liver, lung, kidney and testis [130]. Furthermore, the use of a heterologous viral promoter CMV in the majority of vascular gene transfers causes systemic organ toxicity resulting from unrestricted transgene expression [131]. Retargeting of vectors and use of tissue specific promoters offers an enhanced safety profile by reducing ectopic expression in vital organs including the liver and lungs.

4.3.2. Endovascular gene delivery

Endovascular catheter-based gene delivery allows localization of vectors to the vessel wall and has the advantage that smaller quantities of viral vectors can be used when compared to those used in systemic delivery. The localized delivery minimizes widespread bio-distribution of vectors and simultaneously increases the local vector concentration. Several catheters are used for vascular gene delivery [132], and the efficiency of gene transfer depends on multiple physical parameters during the delivery process, including balloon pressure, vessel wall exposure time, concentration, and injection force [133]. Diffusive balloon catheters that include double balloon, channel, microporous and hydrogel balloons, facilitate passive diffusion of the vector to reach only the innermost layers of the artery (intima and inner media) [134]. Although this system has the advantage of causing relatively minor damage to the vessel media and intima, the major drawbacks include tissue ischemia caused due to blood flow blockage following balloon inflation and relatively low gene transfection rates owing to the short exposure time to the vessel wall. The pressure-driven balloon catheters [135], like the circumferential needle injection balloon catheter and the porous balloon catheter, are thought to efficiently deliver vectors to the deeper medial and adventitial layers of the artery compared to passive diffusion catheters, but they increase the risk of vascular injury. Damage to the endothelial lining promotes SMC proliferation and may lead to restenosis. The localized vascular injury can also cause increased inflammatory response. Iontophoretic catheters, a mechanically assisted injection catheter, enhance the vector penetration across the EC lining by generating an electrical current gradient to drive charged or hydrophilic molecules as deep as the adventitial layer of the artery wall, but depends on the charge, size, and concentration of the delivered compound [136]. Despite the theoretical aspects, in most cases of catheter-based gene transfer the vector is not distributed to the target vessels but to the region of tissue surrounding the target vessel or into the systemic circulation.

Gene eluting stents are attractive alternatives for localized gene delivery as they provide a platform for prolonged gene elution and efficient transduction of opposed arterial walls, especially in the treatment of in stent restenosis [132]. Local delivery of naked plasmid DNA encoding for human VEGF-2 via gene-eluting stent could decrease neointima formation while accelerating re-endothelialization in rabbit model [137]. Stents coated with lipoplexes containing eNOS plasmid accelerated re-endothelialization in hypercholesterolemic rabbits [138]. The same research group also demonstrated successful Ad and AAV delivery to the vessel wall by gene eluting stents with no systemic dissemination of the viral vectors [139]. Stents are often coated with synthetic or naturally occurring biopolymers for prolonged release of the gene to the vessel wall [140]. Recently, fully biodegradable stents have shown great promise in the treatment of peripheral arterial disease [141]. A combination approach of therapeutic gene delivery and fully biodegradable stents would be a novel approach to gene therapy.

4.3.3. Perivascular gene delivery

In endovascular approach, most catheters require prolonged total vascular occlusion for efficient gene delivery to the vasculature increasing the risk of ischemia. Delivery of genes di-

rectly into the adventitia bypassing intima and media may facilitate relatively rapid and efficient delivery compared to endovascular approaches [132]. The advantages of perivascular gene transfer are that the blood flow and endothelium are not disrupted and the placement of vector particles within tissues will result in enhanced local transduction efficiency compared to that achievable by endoluminal delivery [142]. Moreover, the local gene delivery through this 'outside in' approach has received increased attention due to important findings on the capacity of adventitia to influence neointima formation and vascular remodeling [143]. Localized adventitial delivery of a replication-deficient Ad construct containing a fibroblast-active promoter with the gp19ds portion of NADPH inhibitor was effective in reducing overall vascular superoxide anion O_2^- and neointima formation after angioplasty in rat common carotid artery [144]. Shneider *et al.* [145] showed that the infusion of Ad vectors into the carotid artery adventitia achieved recombinant gene expression at a level equivalent to that achieved by means of intraluminal vector infusion. Further, perivascular approach has been reported to minimize the pro-inflammatory effects of Ad vectors [145]. Adventitial gene delivery are also reported to be performed with silastic or biodegradable collars [146] which act as reservoirs of the vector.

The endovascular access is comparatively difficult in the case of coronary arteries, and the numerous side branches will also permit the run-off of the infused volume. An alternative delivery approach for coronary arteries is the expression of diffusible gene products into the pericardial space surrounding the heart and coronary arteries [147]. Transvascular needle injections of Ad vectors to the adventitia and perivascular tissue of coronary arteries have also been reported [148].

4.4. Immunological barriers to gene transfer

The immune system has evolved to eliminate foreign material and therefore, constrains the successful use of gene-replacement therapy based on viral vectors. There are several reports that suggest innate and adaptive immune responses to gene transfer [149, 150]. The vector dose, the route of administration, the nature of the transgene, and host-related factors responsible for inter-individual variability influence the immune response [151]. The early responses involve mechanisms that include the detection of pathogen-associated molecular patterns (PAMPs) present on the viral structural proteins containing the transgene by pattern recognition receptors (PRRs) on cells of the innate immune system (i.e., macrophages and dendritic cells) and the subsequent elaboration of pro-inflammatory cytokines that can up-regulate later adaptive immune responses [152]. The most studied family of PRRs are the toll-like receptors (TLRs), of which TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 have been implicated in initiating inflammatory responses to viruses [153]. The adaptive responses can include: the generation of antibodies to the transgene delivery vehicle compromising vector administration, or the generation of antibodies to the transgene product which nullifies transgene expression, or cytotoxicity to vector and/or transgene product which leads to the loss of transduced cells. It also results in a CD8⁺ memory T cell response that thwarts further efforts to use the same vector or transgene.

Ad vector particles can elicit strong innate and adaptive immune responses. The interplay of both systems activates CD4+ and CD8+ T cells and B cells as well as facilitates the induction of transgene-specific immune responses. The innate immune responses after systemic administration of Ad vectors are due to several processes: complement system activation, anaphylotoxin release, macrophage activation, release of cytokines and chemokines, including Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- α , macrophage inhibitory protein-2, and RANTES (regulated and normal T cell expressed and secreted); EC activation, generalized transcriptome dysregulation in multiple tissues, activation of macrophages and dendritic cells, mobilization of granulocyte and mast cells, and thrombocytopenia [154]. These responses are due to activation of multiple PRRs including RIG-I-like receptors and Toll-like receptors: TLR-2, TLR-4 and TLR-9 [155]. *In vivo* administration of higher doses of Ad vectors can result in one or all of these innate responses or may even lead to mortality in small animal models [156]. Ad infection of ECs is followed by expression of adhesion molecules such as ICAM-1 and VCAM-1 leading to increased leukocyte infiltration within transduced tissues [157]. Kupffer cells, the resident macrophages of the liver, rapidly scavenge and eliminate Ad5-based vectors from the circulation in mice [158], and this interaction contributes to the induction of pro-inflammatory cytokines and chemokines [159]. It has been reported that increasing the dose of Ad vector would probably fail to increase transgene expression, as the CAR adenoviral receptors would become saturated; in addition, the higher dose would induce a stronger inflammatory response responsible for increased elimination of the infected cells expressing the transgene [151].

Ad-based gene transfers can be hindered due to adaptive immune responses to the virus or the transgene it encodes. Ad viruses can induce a cytotoxic T-cell response as well as infiltration by CD4+ and CD8+ T cells. The mechanism involves internalization and priming by dendritic cells of capsid antigens associated with Class II Major histocompatibility complex (MHC) antigens, presentation of these antigens to CD4+ T cells, which become activated, and in turn CD8+ T cell activation by these CD4+ T cells [151]. These adaptive immune responses can limit the duration of transgene expression, and/or limit the ability to re-administer the vector.

Development of new large capacity or gutless (devoid of all viral genes) vectors [160] or modification of capsid sequences [161] are a few of the various strategies devised to reduce the immunogenicity of the Ad viral vectors. Adaptive immunity against these vectors has been substantially reduced through the development of helper-dependent Ad vectors that contain no Ad genes. However, these gutless Ad vectors can efficiently transduce antigen presenting cells (APCs) [162], which readily triggered innate immune responses and further augmented the induction of adaptive immune responses to the transgene product. This problem led to the introduction of tissue-specific promoters in gutless Ad vectors to restrict transgene expression in target cells but not in APCs [162]. Genome modification, capsid modification by Ad capsid-display of immuno-evasive proteins, chimeric Ad vectors and Ad vectors derived from alternative Ad serotypes are few techniques adopted for eluding Ad vector immunity [161]. The tropism modification strategies for targeted gene delivery using Ad vectors have been extensively reviewed [163]. Another method to decrease the im-

immune response is to modify the route of delivery of the vector. In the adventitial delivery of Ad vectors to rabbit carotid arteries, recombinant gene expression was achieved at a level equivalent to that achieved by intraluminal vector infusion. Despite the generation of a systemic immune response, adventitial infusion had no detectable pathologic effects on the vascular intima or media [145]

Pre-existing immunity due to neutralizing antibodies against endemic Ad serotypes in human populations can contribute to pre-existing Ad specific adaptive immune responses [154]. These cellular responses may be more challenging than humoral immune responses, as these cellular adaptive immune responses to Ads have been shown to recognize multiple diverse, cross-clade Ad serotypes subsequent to exposure to only a single Ad serotype [154]. Arterial gene transfer with type 5 Ad vectors did not cause significant levels of gene expression in the majority of humans. Both immune-suppression and further engineering of the vector genome to decrease expression of viral genes show promise in circumventing barriers to Ad-mediated arterial gene transfer [164].

The innate immune response to the AAV capsid has received limited attention due to the minimal responses that AAV2 elicits [162]. According to recent reports by Herzog and others [165], innate immune system also plays important roles in activation of immunity by AAV mediated gene transfer, both in inducing the initial response to the vector and in promoting a deleterious adaptive immune responses. The initial innate immune responses were mediated by the TLR9-MyD88 pathway via a traditional NF- κ B pathway to induce type 1 interferon production. Subsequently, alternative NF- κ B pathway is triggered, prompting adaptive immune responses [166]. *In vivo*, intravenous injection of AAV-lacZ rapidly induces the expression of messenger RNAs (mRNAs) for the cytokines TNF- α , RANTES, interferon- γ -induced protein 10, macrophage inflammatory protein(MIP)-1 β , monocyte chemotactic protein-1, and MIP-2. However, this effect lasts only 6 h, compared to more than 24 h with Ad infection [151]. The adaptive cell-mediated response is far weaker with AAV vectors than with adenoviral vectors probably due to the inability of AAVs to efficiently infect APC, including dendritic cells and macrophages. AAV vectors may be capable of infecting immature dendritic cells, but only when large doses of vector are used. In addition, even though a modest amount of dendritic cells are present at sites of AAV infection *in vivo*, they usually fail to induce a T-cell response of sufficient magnitude to eliminate the infected cells and, therefore, to decrease the duration of transgene expression [151].

Cytotoxic T-cell responses to AAV capsid antigen especially in patients with pre-existing neutralizing antibodies against AAV remain a major road block to achieve persistent therapeutic correction for clinical application. Natural, asymptomatic AAV infection in humans is common, and it estimates that up to 80% of humans possess neutralizing antibodies to some AAV serotypes, especially AAV-2 [167]. Recently, multiple serotypes of AAV in addition to AAV2 have been developed; these serotypes carry different capsid proteins and exhibit different tropism towards different organs [18]. However, changing serotypes may only lead to partial success due to the strong conservation of immune-dominant capsid epitopes in AAVs. In patients with high titers of neutralizing antibodies to gene therapy vectors such as AAV and Ad vectors, IgGs can be removed from blood by plasmapheresis, double filtration

plasmapheresis and immune-absorbant plasmapheresis before gene transfer procedure to increase transduction rates of target tissues [168].

Plasmids alone or in combination with naked bacterial DNA can stimulate innate immune responses [152]. Plasmids, composed chiefly of bacterial DNA, contain far greater amounts of unmethylated CpG motifs than do the DNA in eukaryotic cells. DNA devoid of CpG motifs does not induce proinflammatory cytokine synthesis by macrophages *in vitro*. TLR 9 recognizes the unmethylated CpG motifs in immunostimulatory sequences of bacterial DNA which activate the cells responsible for innate immune responses (for example macrophages) after penetration of bacteria into the body [169]. Indeed, elimination or methylation of these sequences could be a method for suppressing the inflammatory response induced by unmethylated CpG sequences in plasmids [168].

5. Conclusion

An enormous amount of research has been done in the past few decades on the choice of the therapeutic gene, vectors and delivery approaches for effective vascular gene transfer. The low efficiency of gene transfer to vascular tissues still remains a major drawback.. Of the several approaches used so far, Ad-mediated gene transfer has been found to be the most efficient when compared to other methods. However, gene transfer using viral vectors has often caused ectopic expression and also an increased immunological response. The use of tropism modified vectors and plasmids with cell specific promoters are solutions for reducing the ectopic expression. Using “gutless” viral vectors devoid of the immunogenic regions of viral plasmid is an attractive option to reduce the immunologic response, but we have to wait for more *in vivo* data using these third-generation vectors to reach a conclusive result [160]. Non-viral methods have more barriers to overcome to successfully transfect the cell; however, with the advent of innovative technologies like nanobots [170], stimuli responsive polymers [171], novel erythrocyte based carriers [172], magnetically targeted delivery [173] and focused *in vivo* plasmid DNA delivery to the vascular wall via intravascular ultrasound destruction of microbubbles [174]; we expect enhanced transgene expression in vascular cells in future studies. This will also be a possible solution to tackle with the immune response associated with the viral vectors. Site specific biodegradable stent based gene delivery approach [175] and modified percutaneous gene delivery systems offer new opportunities for enhanced gene delivery to vascular cells.

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Gene Therapy for Chronic Pain Management

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1. Introduction

This chapter provides an overview of the main current applications of gene therapy for chronic pain in what concerns animal studies and putative clinical applications. The value of gene therapy in unravelling neuronal brain circuits involved in pain modulation is also analysed. After alerting to the huge socioeconomic impact of chronic pain in modern societies and justifying the need to develop new avenues in pain management, we review the most common animal studies using gene therapy, which consisted on deliveries of replication-defective viral vectors at the periphery with the aim to block nociceptive transmission at the spinal cord. Departing from the data of these animal studies, we present the latest results of clinical trials using gene therapy for pain management in cancer patients. The animal studies dealing with gene delivery in pain control centres of the brain are analysed in what concerns their complexity and interest in unravelling the neurobiological mechanisms of descending pain modulation. The chapter will finish by analysing possible futures of gene therapy for chronic pain management based on the development of vectors which are safer and more specific for the different types of chronic pain.

2. Chronic pain: A burden for modern societies

Pain is not easy to define since it is a highly subjective experience. The more consensual definition of pain was provided by the International Association for the Study of Pain (IASP) and states that *“Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”* [1]. Acute pain is important as an alert signal to potentially threaten situations (internal or external to the organism) and it is important for survival. Acute pain may progress to chronic pain which, according to IASP, is the pain that lasts more than 3 months and persists beyond the normal tissue healing time [2].

Chronic pain may be divided into "nociceptive" and "neuropathic" [3]. Nociceptive pain is caused by activation of nociceptors, the thin nerve fibers which convey nociceptive input from the periphery to the spinal cord. Neuropathic pain is caused by malfunction or damage of the nervous system. Neuropathic pain is frequently difficult to treat being associated to spontaneous pain, exaggerated responses to nociceptive stimuli (*hyperalgesia*) and nociceptive responses to stimuli which are usually non-nociceptive (*allodynia*).

The number of people affected by chronic pain is increasing due to multifactorial causes such as increasing aging of the population. In Europe, about 20% of people suffer from moderate to severe chronic pain [4]. In the United States the prevalence of chronic pain ranges from 2% to 40%, with a median of 15% [5], which cost the country 560 to 635 million dollars [6]. People suffering from chronic pain are less able to walk, sleep normally, perform social activities, exercise or have sexual relations. Chronic pain strongly affects the productivity. About 60% of chronic pain patients are unable or less able to work, 19% lost their jobs and 13% change jobs due to their pain [6]. Chronic pain is associated to several co-morbidities, namely depression and anxiety [6]. Besides all of these indirect costs, chronic pain is a burden due to direct costs of pain management. Despite major investments in basic and clinical pain research, the available analgesics remain considerably unchanged during the last decades. Opioids are useful to manage several pain types but they have a modest efficacy in several pain conditions (e.g. neuropathic pain). Furthermore, long term treatments with opioids frequently induce severe off-target effects, like nausea, constipation and addiction [7]. Intractable pain remains a clinical problem and a drama for the patients and their families [8]. During the last decade, pain clinicians and pain researchers were challenged to search for alternatives to conventional pain treatment, which should be more specific and sustained than conventional analgesics. Gene therapy outstands as a powerful technique to overcome some current problems of chronic pain treatments.

Neurobiological research in the pain field provided solid information regarding the transmission and modulation of nociceptive information from the periphery to the brain, where a pain sensation is produced (Fig. 1). Nociceptive signals are conveyed by primary afferent fibers from peripheral organs, like the bladder or muscles, to the spinal cord. This is the first relay station involved in the modulation of nociceptive information namely by local inhibitory interneurons that use opioid peptides or aminoacids (γ -aminobutyric acid-GABA- and glycine). Nociceptive information is then transmitted supraspinally, namely to the thalamus, and to several brainstem areas, where additional modulation of the nociceptive signal occurs. The thalamo-cortical pathway ensures that the nociceptive information reaches the somatosensory and prefrontal cortices, where the nociceptive signal is finally perceived as a pain sensation [9, 10]. Some brain areas which directly or indirectly receive nociceptive information from the spinal cord are also involved in descending pain modulation. Both inhibition and facilitation may occur and chronic pain may derive from a reduction of the former and enhancement of the latter [9, 11]. This neurobiological knowledge has been used to design gene therapy studies for chronic pain, namely to choose the somatosensory system areas and neurotransmitters/receptors to be targeted in order to block nociceptive transmission.

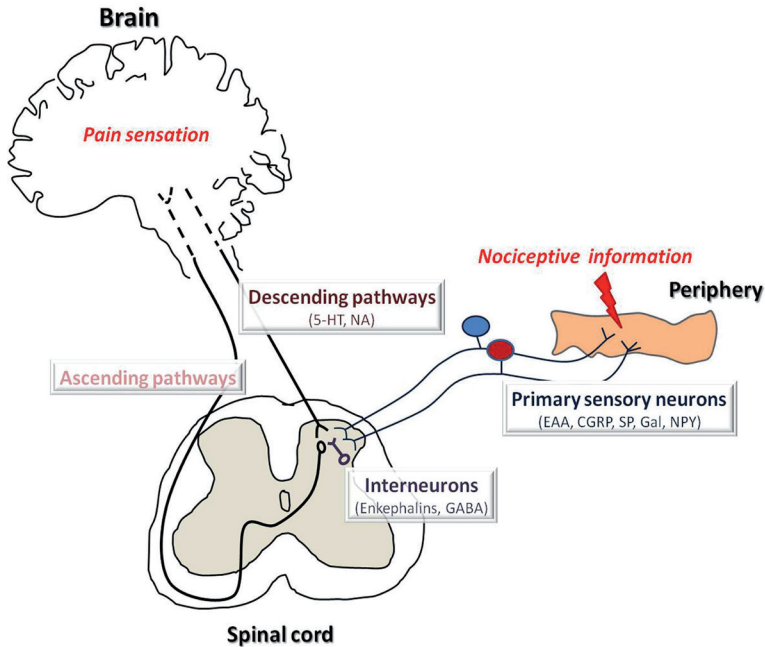


Figure 1. Schematic diagram of pain pathways involved in pain transmission and modulation. **Nociceptive information** is transmitted from the periphery to the spinal dorsal horn by primary sensory neurons. At the spinal level, these neurons transmit nociceptive information to second order neurons ("Ascending pathways") through the release of neurotransmitters like the excitatory amino acids (EAA) glutamate and aspartate, calcitonin gene-related peptide (CGRP), substance P (SP) galanin (Gal) and neuropeptide Y (NPY). In the brain, the nociceptive information is then perceived as a **pain sensation**. The transmission of nociceptive information at the spinal level is modulated by interneurons (mainly inhibitory) through the release of opioid peptides and GABA and also by supraspinal descending neurons ("Descending pathways") through the release of serotonin (5-HT) and noradrenaline (NA). Descending pathways may inhibit or enhance nociceptive transmission from the spinal cord.

Gene therapy is an especially versatile tool for chronic pain management since it is based in a triad of controllable parameters: the vector, the transgene and the promoter. By knowing the neurobiological features of each chronic pain type, namely the neurotransmitters and receptors affected, it is possible to design gene therapy strategies based on the best combination of vectors, transgenes and promoters. As to **vectors**, gene therapy for pain uses mainly "vehicles" which have a "certified" experience in infecting neurons, namely replication-defective forms of viruses. Non-viral vectors have seldom been used in gene therapy studies for pain but their transduction efficiency and specificity are much lower than those of viral vectors. Some of these vectors have the ability to migrate retrogradely (i.e., contrary to the direction of nerve impulse) which is very useful to target neurons that are located in structures of difficult surgical

access. A good example is the application of replication-defective forms of Herpes Simplex Virus type 1 (HSV-1) at the periphery (e.g. the skin) to transduce neurons at the spinal ganglia (dorsal root ganglia-DRGs), which are difficult to access due to their bone protection. Regarding the **transgenes** to include in the vectors for gene therapy of pain, it is possible to increase the expression of neurotransmitters and receptors involved in nociceptive inhibition (e.g. opioids), neurotrophic factors or substances with anti-inflammatory properties. Finally, and in what concerns the **promoters**, it is possible to choose those that restrict transgene expression to a cell type, such as a neuron or a glial cell, or even target selective neurochemical neuronal populations. Examples of neuron-specific promoters are synapsin I, calcium/calmodulin-dependent protein kinase II, tubulin alpha I and neuron-specific enolase [12]. Some possibilities of controlling the vectors, transgenes and promoters will be discussed in the next two sections using gene therapy in animal models.

3. Gene therapy targeting the spinal cord in animal pain models

One of the main advantages of experimental gene therapy studies is that they can be performed using several pain models. This is important since each pain type may induce specific changes in neuronal circuits devoted to the transmission and modulation of nociceptive transmission [13]. Studies of gene therapy for pain have used clinically relevant models of inflammatory [14-22] and neuropathic pain [23-34]. In a much lower incidence, models of acute [35-38], post-operative pain [39] and cancer [40] pain have been used in experimental gene therapy studies. The large majority of studies were performed in pain models affecting the limbs or the trunk, in the latter case being of visceral origin [22, 37]. Two studies used gene therapy to block nociceptive transmission coming from the head/face in pain models that reproduces some types of craniofacial pain, like trigeminal neuralgia [41] or temporomandibular joint disorders [42].

Gene therapy studies for pain in animal models may be divided in studies targeting the spinal cord (Table 1) and studies directed to pain control centres located in the brain (Table 2). Studies directed to the spinal cord mainly aim to manipulate the expression of transgenes in order to block the transmission of nociceptive input at the spinal dorsal horn (Table 1). Most of the spinal cord studies using gene therapy for pain elected HSV-1 as the most suitable vector, due to its natural affinity to the neuron and its ability for retrograde transport [43]. HSV-1 has the additional advantage over other vectors of carrying multiple transgenes or large transgenes and not integrating in the host genome, which reduces the possibility of mutagenic events [44, 45]. After application of replication-defective forms of HSV-1 at the periphery in order to transduce DRG neurons (or trigeminal ganglion neurons), delivery of the transgene product by the spinal branch of transduced neurons at the spinal dorsal horn induced analgesia in several rodent models of pain (Table 1). Gene therapy in animal models of craniofacial pain [41, 42] aimed to release the transgene products at the level of the spinal trigeminal nucleus and this structure is homolog of the spinal cord, which prompted to include these studies in the section devoted to spinal cord studies.

Pain models	Gene product	Inoculation	References
Herpes Simplex type 1			
Acute pain	Pre-proenkephalin	Subcutaneous	[35]
Inflammatory pain	Pre-proenkephalin A	Subcutaneous	[14]
Neuropathic pain	Pre-proenkephalin A	Subcutaneous	[41]
Cutaneous hyperalgesia	Pre-preproenkephalin	Subcutaneous	[36]
Bladder hyperactivity	Pre-preproenkephalin	Bladder wall	[37]
Inflammatory pain	Endomorphin-2	Subcutaneous	[15]
Neuropathic pain	Endomorphin-2	Subcutaneous	[23]
Neuropathic pain	IL-4	Subcutaneous	[24]
Neuropathic pain	sTNFRs	Subcutaneous	[25]
Neuropathic pain	GAD	Subcutaneous	[26, 47]
Chronic pancreatitis	Pre-proenkephalin	Pancreas surface	[22]
Inflammatory pain	Na _v 1.7 antisense	Subcutaneous	[16]
Incision pain	Pre-proenkephalin	Subcutaneous	[39]
Cancer pain	Pre-proenkephalin	Subcutaneous	[40]
Adenovirus			
Inflammatory pain	GAD	Trigeminal ganglion	[42]
Neuropathic pain	IL-10	Intrathecal	[27]
Inflammatory pain	β -endorphin	Intrathecal	[17]
Neuropathic pain	IL-2	Intrathecal	[28]
Adeno-associated vectors			
Neuropathic pain	IL-10	Intrathecal	[29]
Neuropathic pain	shGCH1	Intrathecal	[30]
Neuropathic pain	Prepro- β -endorphin	Intrathecal	[31]
Inflammatory pain	μ -opioid receptors	DRG	[18]
Lentivirus			
Neuropathic pain	GDNF	Intraspinal	[32]
Neuropathic pain	NF κ B Repressor	Intraspinal	[33]

Table 1. Summary of experimental studies using viral vectors for gene transfer to the spinal cord.

As to the transgenes included in the HSV-1 vectors, opioid peptides or their precursors largely prevail due to their well-known ability to block nociceptive transmission at the spinal cord. HSV-1-based delivery of opioids has additional advantages over classic opioids, namely by

being deprived of major side-effects and preventing tolerance after repeated administrations of the vector [46]. Furthermore, opioid-based gene therapy can be very powerful in inducing analgesia if combined with administration of very low doses of classical opioids [46]. Besides opioid peptides, other transgenes were included in the HSV-1 vectors constructs. A transgene that increases the levels of the inhibitory neurotransmitter GABA, namely by overexpressing its synthesizing enzyme glutamate decarboxylase (GAD), induced analgesia in neuropathic pain models [26, 47]. HSV-1 based vectors have also been used to deliver transgenes that overexpress anti-inflammatory interleukins [24, 48] or the soluble receptor for tumor necrosis factor- α (TNF- α), which act as an antagonist of TNF- α in order to block its role as a pro-inflammatory mediator [25, 49]. A decrease in the levels of the α subunit of the voltage-gated sodium channel 1.7 (Nav 1.7) was also achieved using HSV-1 constructs but with the transgene inserted in antisense orientation [16].

Other viral vectors, namely adenoviruses, adeno-associated viruses and lentiviruses have been used to target the spinal cord, but unlike HSV-1 vectors which have been administered at the periphery following its natural route of retrograde transport to DRG neurons, these vectors were either directly injected into DRGs or trigeminal ganglion neurons, intrathecally or intraspinally (Table 1). The transgenes included in adenoviruses, adeno-associated viruses are similar to those used in HSV-1 vectors, namely opioids [17, 31], interleukins [27-29] and GAD [42]. Adeno-associated vectors have also carried transgenes that overexpress μ -opioid receptors [18] or block the expression of GTP cyclohydroxylase (GCH1) using small hairpin RNAs [30]. GCH1 is the rate-limiting enzyme of an essential co-factor for nitric oxide synthase (NOS), which modulates nociceptive transmission. Finally, lentiviral vectors have also been used in gene transfer studies directed to the spinal cord. Based on its ability to restrict transduction to the injection site, lentiviral vectors have been administered intraspinally in the dorsal horn to increase the levels of a neurotrophic factor (glial-derived neurotrophic factor, GDNF) [32] or decrease the expression of Nuclear Factor κ B (NF κ B), which regulates cellular inflammation responses [33]. In the latter study, microdelivery of an HIV pseudotyped lentiviral vector into the spinal dorsal horn led to a preferential transgene expression in glial cells. This shows that, besides the promoter, pseudotyping the vector is a way of directing transgene expression and glia is an important target in pain, inasmuch that chronic pain is associated to the activation of glial cells which produce algogenic mediators that exacerbate pain, namely NF κ B. All of these approaches showed considerable analgesic efficacy and reduced side effects.

4. Gene therapy targeting for pain: The challenge of targeting pain control circuits in the brain

Abnormal descending pain modulation from the brain is a common feature of several chronic pain conditions, namely those characterized by widespread pain, like fibromyalgia, which derive from impairments in descending pain inhibition [50]. Studies with gene delivery into the brain (Table 2) are much scarcer than spinal cord deliveries. This is due both to the higher difficulty of surgical approaches to deliver the vectors into the brain and challenges to

manipulate the complex brain neuronal circuits involved in pain modulation. The spinal cord constitutes a less invasive delivery route when the aim is to manipulate descending modulatory pathways (Fig. 1). This delivery route was recently explored by injecting intraspinally an adenovirus vector targeting the expression of a potassium channel into noradrenergic pontospinal neurons, which decreased the activity of those pontospinal neurons and induced hyperalgesia [51]. These experiments confirm the pain inhibiting role of the noradrenergic projections to the spinal cord [52].

Pain models	Gene product	Delivery	References
Acute pain	Proenkephalin	Medullary dorsal reticular nucleus (DRt)	[55]
Neuropathic	Tyrosine Hydroxylase antisense	Medullary dorsal reticular nucleus (DRt)	[34]
Inflammatory	Proenkephalin	Medullary ventral reticular nucleus (VLM)	[19]
Inflammatory	Proenkephalin	Medullary dorsal reticular nucleus (DRt)	[20]
Acute pain	GAD	Insular Cortex	[38]
Inflammatory pain	Preproenkephalin	Amygdala	[21]
Acute and Inflammatory pain	NMDA antisense	Medullary nucleus of the solitary tract	[56]
Inflammatory and neuropathic pain model	Potassium channel (hKir _{2.1})	Pontospinal noradrenergic neurons	[51]

Table 2. Summary of animal studies using viral vectors for gene transfer to pain control centers in the brain.

Gene transfer in the brain used almost exclusively HSV-1 vectors to overexpress opioid peptides [19-21, 38] and, in a much more limited extent, GAD [38]. Our research group has a large experience in gene transfer to pain control centres at the medulla oblongata, namely the dorsal reticular nucleus (DRt), the caudal ventrolateral medulla (VLM) and the nucleus of the solitary tract (NTS). These areas were elected based on the extensive neurobiological knowledge of their role in pain modulation [53, 54]. Overexpression of opioid precursors in the DRt and VLM induced analgesia in acute pain tests and models of sustained or chronic inflammatory pain [19, 20, 55]. Brain areas involved in pain control and which are of easier neurosurgical access are the amygdala and the rostral agranular insular cortex. Overexpression of opioid precursors in the central amygdalar nucleus [21] or GAD in the rostral agranular insular cortex induced analgesia in acute pain models [38]. Lentiviral vectors were delivered to the NTS, an area which is crucial in pain and cardiovascular integration, to decrease local expression of N-methyl-D-aspartate (NMDA) receptor, a key receptor for the action of glutamate, and this approach was shown to decrease acute and inflammatory pain [56]. Since glutamate, is the most ubiquitous mediator of excitatory synaptic transmission in the central nervous system

and NMDA receptors are also expressed by glial cells, the effects of gene therapy were restricted to NTS neurons by using the rat synapsin promoter.

There is a puzzling difference between gene therapy studies using HSV-1 vectors at the periphery or the brain. Whereas the ability for HSV-1 to migrate retrogradely is the main feature of studies at the periphery, the migration of HSV-1 in the brain is seldom evaluated. This can confound the effects of gene therapy on pain responses since the effect may derive from transduction of neurons that project to the injected area, and not at the targeted area itself. Our research group has pioneer work in studying the dynamics of HSV-1 migration in the brain after injections of the vector in pain control centres of the medulla oblongata, namely the caudal medulla oblongata (VLM) and the dorsal reticular nucleus (DRt). After injections of a HSV-1 vector expressing the lacZ reporter gene, under control of the human cytomegalovirus promoter (hCMV), in pain control centres of the medulla oblongata, migration in VLM and DRt afferents was detected [19, 55] (Fig. 2). However, not all the brain afferents of the VLM and DRt exhibited β -galactosidase (β -gal), the product of *lacZ* expression. For example, the amygdala and the cortex, which are important VLM and DRt afferents [57, 58] did not show neurons expressing β -gal.

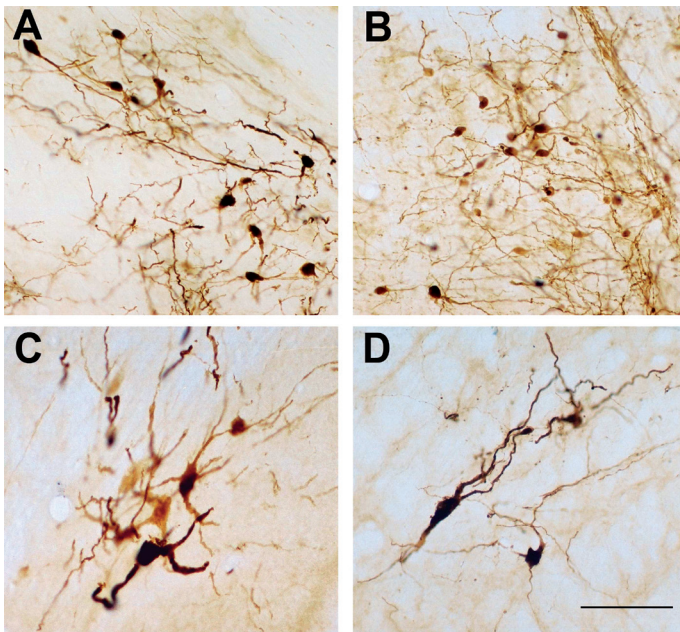


Figure 2. Dynamics of HSV-1 migration in the brain after injection into the DRt. Photomicrographs of β -gal positive neurons in the cerebellum (A), the parabrachial complex (B), the locus coeruleus (C) and the VLM (D) at 7 days post-injection Scale bar D: 100 μ m (photomicrographs A-C are at the same magnification).

Although it could be argued that this is due to lack of activity of the hCMV promoter in amygdalar and cortical neurons, other studies showed that hCMV is active in those neurons [21, 59]. These results rather point to a selective uptake of HSV-1 vectors injected in the brain parenchyma, probably due to interactions between neuronal receptors and glycoproteins of the HSV-1 envelope. By carefully mapping the brain areas exhibiting retrograde transport after HSV-1 injections in the brain using immunohistochemical detection of the gene reporter and in situ hybridization against the DNA of HSV-1, the problems of affecting brain afferents of the injected area can be circumvented.

The selective migration of HSV-1 in the brain can be a useful feature of the vector. After establishing the dynamics of the migration of HSV-1 in the brain after injection into the DRt (a facilitatory pain control centre of the brain), we used a tissue specific promoter (tyrosine hydroxylase-TH) to direct the expression of the vector to the noradrenergic afferents of the DRt (Fig. 3). Based on the analgesic effects of the administration of $\alpha 1$ -adrenoreceptor antagonists into the DRt, the TH transgene was inserted in antisense orientation into the vector in order to decrease the levels of noradrenaline in the DRt [34]. A sustained analgesic effect was achieved in a model of neuropathic pain, which reproduces clinically relevant features of neuropathic pain. The fact that the analgesic effects were so long, lasting for 2 months with a single vector injection, and reversed several pain modalities, indicates that targeting pain control centres of the brain needs to be considered both in animal and pre-clinical studies.

5. Gene therapy for chronic pain at the bedside: Human studies

The translational perspectives of the studies summarized in section 2, namely those using replication-defective HSV-1 vectors, favoured the approval of clinical trials for gene therapy for chronic pain. An important reinforcement of the proof-of-concept for the potential utility of HSV-based vector in rodent pain models was provided by equivalent studies performed in primates [36]. These studies were important since the translational perspectives of the rodent results were questioned for several reasons, such as the larger size of dermatomes of humans. The first clinical trial of gene therapy for pain was a safety and dose-escalation Phase I study in ten patients with mild to severe intractable pain due to terminal cancer [60]. The protocol consisted in the administration directly in the pain-reporting area of an HSV-1 replication-defective vector containing the transgene of the precursor of enkephalin [61]. A dose-dependent analgesic effect was demonstrated with a reduction of pain scores lasting for at least 2 weeks and with no adverse effects. These encouraging results prompt to implement a Phase II trial in a larger cancer population and the study includes placebo controls, evaluation of the effects of reinoculation of the vector and assessment of the maximal dose [45, 62].

The progress of the clinical trials for cancer pain opened avenues to test gene therapy to block nociceptive transmission in the spinal cord in other pain conditions, such as painful diabetic neuropathy. This pain type, which is increasing to the pandemic occurrence of diabetes, is difficult to treat with conventional analgesics and only about one third of the patients achieve a 50% pain reduction beyond the placebo effect [63]. A clinical trial has recently been approved

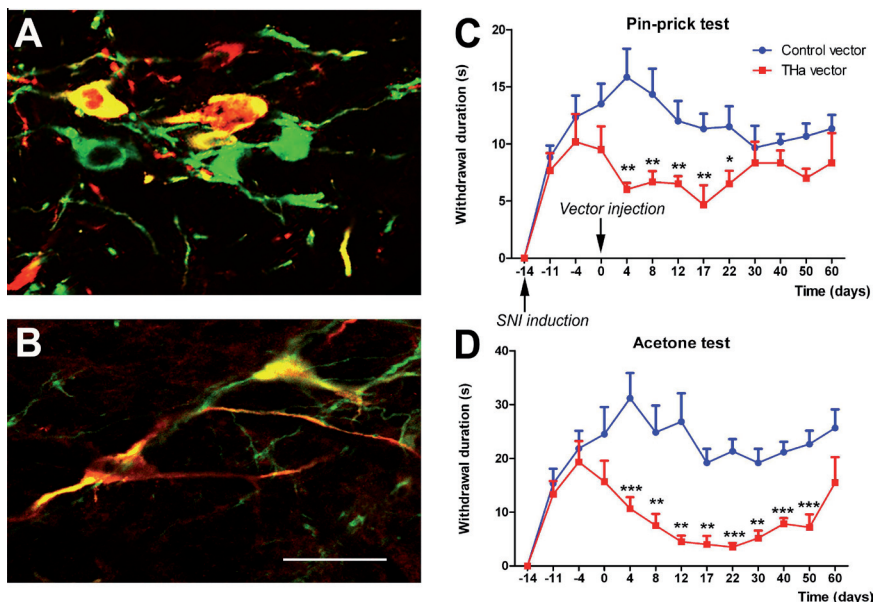


Figure 3. HSV-1 injected at the DRT transduces noradrenergic afferents of the nucleus (A, B). Photomicrographs representing double-labeled neurons for β -gal and TH (yellowish) in the locus coeruleus (A) and the A₅ noradrenergic cell group (B). β -gal positive neurons are shown in red and TH positive neurons are shown in green (A). Scale bar in B: 40 μ m (A is at the same magnification). The insertion of TH in antisense orientation into HSV-1 (THa vector) induced analgesia in the spared nerve injury (SNI) model of neuropathic pain (C, D). THa induced a sustained attenuation of mechanical hyperalgesia evaluated by the pin-prick test (C) and cold allodynia evaluated by the acetone test (D). THa and the control vector were injected at time 0, i.e., 2 weeks after SNI induction. Data are presented as mean \pm SEM (n=6 for each group); *P<0.05, **P<0.01, ***P<0.001 THa- vs. control- vector.

to use an HSV-1 vector that overexpress GAD to relief painful diabetic neuropathy [45]. Other therapeutic transgenes are being considered for future clinical trials of gene therapy, namely the overexpression of interleukins [45]. The future of gene therapy for chronic pain in humans will depend on the results of the clinical trials that are currently being performed but the promising results obtained so far indicate that gene therapy will add to the armamentarium of available pain treatments.

The application of gene therapy to block nociceptive transmission at supraspinal levels has been proposed by several pain specialists [64]. However, most experimental studies dealing with gene delivery at the brain were directed to pain control areas of the medulla oblongata, which are of difficult neurosurgical approach since they are in close vicinity to areas involved in the control of vital functions, such as cardiovascular and respiratory controls. Moving the focus of the gene delivery studies to areas that are more easy to approach may be useful namely in the context of widespread chronic pain, such as fibromyalgia and complex regional pain syndrome [65]. This can only be considered after a thorough characterization of the pain control

circuits in the brain namely in what concerns the functional changes induced by the chronic pain condition in order to select the best brain areas to target to maximise the balance between efficacy and risk.

6. Future challenges

The advances of gene therapy in other diseases of the nervous system rather than pain will be crucial to define the future of gene therapy for chronic pain, namely by the improvements in the delivery systems. Studies which improved the efficacy of non-viral vectors already inspired the construction of a non-viral, non plasmid immunologically defined gene expression (MIDGE) vector that overexpress β -endorphin and induced analgesia after injection into inflamed paws by increasing the concentration of β -endorphin in leukocytes [66]. Since chronic pain requires long-term transgene expression, the duration of the activity of promoters needs to be increased. It could be useful to design constructs that are activated only when pain lasts for longer periods and rises over a certain threshold. This would allow treating chronic pain but still preserve acute pain as an alert signal. An interesting possibility could be to control the activity of the promoter using inducible promoters, which have been used in gene therapy studies other than pain. The activity of these promoters can be induced exogenously, for example, by antibiotics. An ingenious idea was recently applied by using a ligand (glycine) which normally is not expressed in DRG neurons but can be administered to activate HSV-1 vectors to express glycine receptors in animal models of somatic and visceral pain [67]. Besides the vectors and the promoters, an election of effective transgenes for chronic pain will be important to define the future of gene therapy for chronic pain. Transgenes for opioid peptides have been overused in gene therapy studies in animal models but long-term treatments with classic opioids may induce pain, a phenomena known as opioid-induced hyperalgesia [68]. By achieving more sustained and strong transgene expression, it is possible that opioid-induced hyperalgesia could also be induced by gene therapy. New transgenes should be considered in future studies of gene therapy for chronic pain. Based on the role of the vanilloid receptor TRPV-1 (Transient Receptor Potential channel Vanilloid 1) as a pro-nociceptive cationic channel involved in pain signalling, and the clinical relevance of desensitization of TRPV1 receptors [69], this may be an important target molecule in the future. By decreasing the expression of protein kinase C-epsilon (PKC), which phosphorylates TRPV1 receptors, it was possible to induce analgesia in animal models [70]. Even more challenging is the possibility of targeting pain control centres in the brain using gene therapy. These studies will continue for large years to focus on animal pain models in order to determine neurobiological effects of chronic pain installation in pain control centres, using gene therapy as a method to prevent those changes. Finally, the emergent new field in pain research of genetics of pain has recently provided data which may explain the higher susceptibility of some persons to develop chronic pain [71]. Due to its versatility and the possibility of direct gene targeting, gene therapy can be the perfect tool to verify if the holy grail of a personalized pain treatment can be implemented.

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List of abbreviations

β -gal- β -galactosidase

DRG- Dorsal root ganglion

DRT- Dorsal reticular nucleus

GABA- γ -amminobutiric acid

GAD- glutamate decarboxylase

GDNF- Glial-derived neurotrophic factor

hCMV- Human cytomegalovirus

HSV-1- Herpes Simplex Virus type 1

IASP- International Association for the Study of Pain

IL₂- Interleukin 2

IL₄- Interleukin 4

IL₁₀- Interleukin 10

MIDGE- Non plasmid immunologically defined gene expression

Nav 1.7- Voltage-gated sodium channel 1.7

NF κ B- Nuclear Factor κ B

NMDA- N-methyl-D-aspartate

NTS- Nucleus of the solitary tract

PKC- Protein kinase C-epsilon

shGCH1- small hairpin RNAs for GTP cyclohydroxylase

sTNFRs- tumor necrosis factor- α

TH- Tyrosine hydroxylase

TRPV-1- Transient Receptor Potential channel Vanilloid 1

VLM- Caudal ventrolateral medulla

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Insulin Trafficking in a Glucose Responsive Engineered Human Liver Cell Line is Regulated by the Interaction of ATP-Sensitive Potassium Channels and Voltage-Gated Calcium Channels

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1. Introduction

Type I diabetes is caused by the autoimmune destruction of pancreatic beta (β) cells [1]. Current treatment requires multiple daily injections of insulin to control blood glucose levels. Tight glucose control lowers, but does not eliminate, the onset of diabetic complications, which greatly reduce the quality and longevity of life for patients. Transplantation of pancreatic tissue as a treatment is restricted by the scarcity of donors and the requirement for lifelong immunosuppression to preserve the graft, which carries adverse side-effects. This is of particular concern as Type 1 diabetes predominantly affects children. Lack of glucose control could be overcome by genetically engineering "an artificial β -cell" that is capable of synthesising, storing and secreting insulin in response to metabolic signals. The donor cell type must be readily accessible and capable of being engineered to synthesise, process, store and secrete insulin under physiological conditions.

The cell type of choice for the gene therapy of diabetes is not the β -cell. β -cells are greatly reduced or absent in people with Type I diabetes because of their autoimmune destruction. This fact will actively work against gene therapists trying to derive surrogate β -cells from

stem cells. There are innumerable theories describing putative mechanisms for preventing a patient's immune system from re-attacking transplanted β -cells, but the fact that the basic processes of islet cell attack have not been fully elucidated makes the search for relevant genes problematic. Thus, the engineering of non-pancreatic β -cells to synthesise, process, store and secrete insulin has several advantages, the most important of which is the ready availability of donor cells. If non β -cells from a diabetic individual can be engineered to produce insulin, then cellular rejection is less likely to occur since donor and recipient are autologous. In pursuit of this goal, hepatocytes have been shown to be suitable target cells for the generation of artificial β -cells [2-9]. Moreover, liver cells that produce insulin may not be prone to autoimmune attack [10]. The suitability of hepatocytes as a β -cell replacement is attributable, in part, to their inherent glucose responsiveness and their embryonic origin from the same endodermal precursor cells as the β -cell. Most importantly, liver cells express the high capacity glucose transporter, GLUT 2 [11], and the high capacity phosphorylation enzyme, glucokinase [12], which constitute the key elements of the "glucose sensing system" that regulates insulin release from pancreatic β -cells in response to small extracellular nutrient changes.

In pancreatic β -cells, a small increase in plasma glucose concentration stimulates significant insulin secretion. Therefore, glucose is the major modulator of β -cell function and this behaviour must be mimicked in insulin-secreting liver cells. In pancreatic β -cells, K_{ATP} channels, which are composed of four sulphonylurea receptor (SUR) subunits and four inwardly-rectifying potassium channel ($K_{IR6.2}$) subunits [13-18], maintain resting membrane potentials and link plasma glucose concentrations to the insulin secretory machinery. The triggering pathway for insulin release begins with the uptake of glucose via the glucose carrier, GLUT2, and an acceleration of metabolism, such that glucose is used to generate ATP. An increase in the absolute intracellular concentration of ATP, with respect to ADP, stimulates the closure of K_{ATP} channels [19, 20]. Potassium conductance of the plasma membrane decreases, allowing a background current to shift the membrane potential away from the equilibrium potential for K^+ , thus depolarising the membrane. Consequently, the pancreatic β -cell is able to translate metabolic signals to electrical signals, the latter regulating insulin secretion. Lack of functional K_{ATP} channels in insulin-secreting NES2Y cells resulted in the unregulated release of insulin, which was restored by expression of both $K_{IR6.2}$ and SUR1 [21].

When depolarisation of the pancreatic β -cell reaches the threshold for activation of L-type ($Ca_v1.3$), and to a lesser extent P/Q ($Ca_v1.2$) and T-type ($Ca_v3.x$) voltage-gated calcium channels, these open allowing Ca^{2+} influx down their electrochemical gradient [22]. The opening of Ca_v channels is intermittent, fluctuating with the membrane potential, therefore generating oscillations in the intracellular (cytosolic) calcium concentration ($[Ca^{2+}]_i$), which, in turn, triggers pulsatile insulin secretion. In β -cells, elevation of $[Ca^{2+}]_i$ occurs via the release of Ca^{2+} from intracellular stores (endoplasmic reticulum, mitochondria and secretory granules) and/or influx of extracellular Ca^{2+} through Ca_v channels [23, 24]. No functional Ca_v channels have been previously described in liver cells, however the presence of an $\alpha 1$ -subunit lacking the voltage sensor has been reported in the rat liver cell line H4IIE [25] and an L-type $\alpha 1$ -subunit has been detected at low levels in rat liver by RT-PCR [26].

For an insulin-producing liver cell to be of maximum benefit *in vivo* it must be capable of rapid responsive secretion of biologically active insulin. This characteristic demands that artificial β -cells process proinsulin to insulin and store it in granules. Our previous studies have shown that the insertion of genes encoding for insulin and the glucose transporter, GLUT2, into the HEPG2 human hepatoma cell line, resulted in synthesis and storage of (pro)insulin in structures resembling the secretory granules of pancreatic β -cells (HEPG2/ins/g), and the near physiological secretion of (pro)insulin in response to glucose [2, 3]. Similar to pancreatic β -cells, HEPG2ins/g cells responded to glucose via signalling pathways dependent upon K_{ATP} channels [27]. Therefore, expression of both insulin and GLUT2 in HEPG2 liver cells appeared to be sufficient for the generation of functional K_{ATP} channels, unlike the parental cell line that required pharmacological stimulation to activate the K_{ATP} channels [28]. It has previously been shown that stable transfection of the insulin gene into the human liver cell line, Huh7 (which endogenously expresses GLUT2), resulted in synthesis, storage, and regulated release of insulin to the physiological stimulus glucose (Huh7ins cells) [7]. Huh7ins cells are more akin to pancreatic β -cells than HEPG2/ins/g cells. They express a range of β -cell transcription factors [7, 29] and possess storage granules that cleave proinsulin to biologically active diarginyl insulin, due to the expression of the proconvertases PC1 and PC2 [7]. As Huh7ins cells also rapidly secrete insulin in a tightly regulated manner in response to glucose, the Huh7ins cells were able to reverse chemically induced diabetes when transplanted into an animal model [7], which HEPG2ins/g cells [3] failed to achieve [Tuch, unpublished results].

This chapter will detail the use of electrophysiological and biochemical techniques to show that Huh7ins cells respond to a glucose stimulus by closure of K_{ATP} channels and activation of Ca_v channels, which is an analogous mechanism to pancreatic β -cells. Patch-clamp electrophysiology of Huh7ins cells yielded current-voltage (I - V) curves that indicated the presence of potassium-selective currents; in contrast, currents recorded from Huh7 cells were non-selective. The presence of functional ATP-sensitive potassium (K_{ATP}) channels and voltage-gated calcium (Ca_v) channels was further validated by measurement of acute insulin secretion by Huh7ins cells in response to pharmacological channel inhibitors and activators and by calcium imaging and patch-clamp electrophysiology experiments. Molecular analyses were used to confirm that the Huh7ins cells express Ca_v and all the subunits of K_{ATP} channels. The secretion of insulin from granules in live Huh7ins cells was revealed by confocal microscopy which allowed visualization of secretion of insulin to a zink probe or an insulin-enhanced green fluorescent protein (EGFP) fusion protein (EGFP-ins). The glucose responsive mechanism that we observed in the Huh7ins cells was the same as that reported for the pancreatic β -cell line, MIN6 [30]. Prior to this study, the physiological interaction of K_{ATP} channels and Ca_v channels had never been shown in liver cells engineered to secrete insulin. As the biochemical properties of Huh7ins cells are akin to those of pancreatic β -cells, engineering hepatocytes in this way opens a promising avenue for the ultimate replacement of the endogenous β -cell function that is lost in Type I diabetes, by modifying a patient's own liver cells to become artificial β -cells. This is the first study that clearly delineates the control of insulin trafficking in a functioning artificial β -cell line that was derived from a human liver cell.

2. Understanding the mechanism by which liver-derived artificial beta cells respond to glucose and pharmacological stimulators and inhibitors of insulin secretion:

The mechanisms by which liver-derived artificial β -cells respond to glucose are poorly understood. Indeed, the majority of engineered insulin-secreting liver cells lack a truly regulated pathway of insulin release [31]. As pancreas and liver are derived from the same endodermal origin, the capacity of liver cells to differentiate into cells bearing pancreatic characteristics is well documented. A number of studies have shown that the expression of β -cell transcription factors in liver cells leads to pancreatic transdifferentiation, glucose-regulated insulin secretion and reversal of diabetes [4-7, 9, 32, 33]. Spontaneous pancreatic transdifferentiation and glucose-regulated insulin secretion have also been shown in dedifferentiated liver cells that express β -cell transcription factors such as the HEPG2ins/g and Huh7ins liver cell lines [3, 7, 9], as well as liver cells that have experienced a metabolic insult such as hepatic oval cells cultured in high glucose [34]. Consistent with this, our laboratory has shown spontaneous pancreatic transdifferentiation in hyperglycaemic rat livers and reversal of diabetes following the delivery of the insulin gene using a lentiviral vector [8]. Other recent studies in our laboratory have employed the H4IIE liver cell line, which does not express β -cell transcription factors and lacks a regulated pathway of insulin release [31]. When engineered to express the β -cell transcription factor *Neurod1* and rat insulin (H4IIEins/ND), H4IIE cells underwent pancreatic transdifferentiation and glucose-regulated insulin secretion from secretory granules. However, when *Neurod1* alone was expressed, an array of β -cell transcription factors and pancreatic hormones were expressed, but glucose-regulated insulin-secretion was not observed [9]. The Huh7 parent cell line, from which the insulin-secreting Huh7ins cells were derived, represents an ideal candidate for the engineering of an artificial β -cell. These cells possess several characteristics inherent to β -cells but not intrinsic to primary hepatocytes, such as the expression of β -cell transcription factors *Neurod1* [7], *Pdx1*, *Nkx2-2*, *Nkx6-1*, *Neurog3* and *Pax 6* [29]. Importantly, however, the process of transfection with insulin resulted in the formation of insulin secretory granules and the development of a regulated insulin secretory pathway [7] as was observed in the rodent H4IIEins/ND cells. Results of a mechanistic microarray analysis comparing Huh and Huh7ins cells following insulin transfection indicated that the formation of secretory granules and the development of a regulated secretory pathway was likely related to a protein interaction or posttranslational effect in combination with increased gene expression of secretory granule proteins such as chromogranin A [29].

2.1. Huh7ins cells possess potassium-selective plasma membrane channels

Huh7 (parental human liver cell line), Huh7ins (parental human liver cell line transfected with human insulin cDNA) [7] were maintained in Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% v/v fetal calf serum (FCS) (Trace Biosciences, Australia) in 5% CO₂ at 37°C. Although of murine origin, MIN6 cells are one of the few β -cell lines that are responsive to glucose in the physiological range, and, accordingly provide an established β -cell-like cell line for comparative purposes [30]. MIN6 cells were grown in DMEM supplemented with 15% v/v FCS (37°C, 5% CO₂). For the Huh7ins cell line, the selective antibiotic G418 (0.55 mg/ml) was added to maintain stable transfectants.

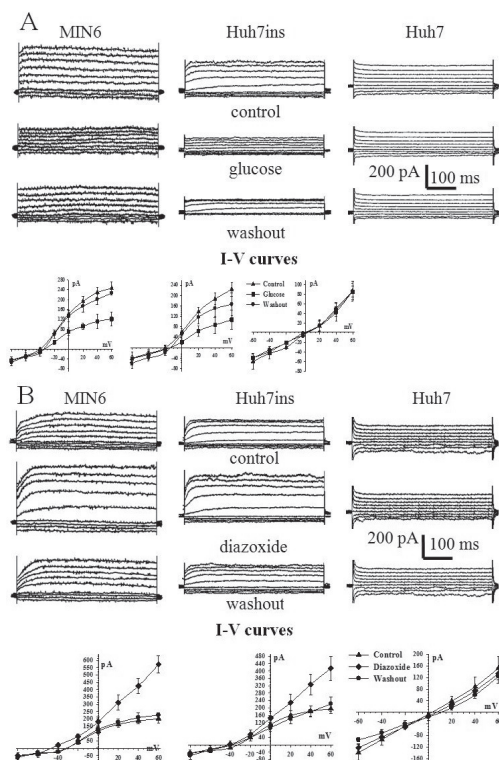


Figure 1. Sensitivity of potassium channels to glucose and diazoxide in Huh7ins cells. The upper three sets of current traces in panels A and B show superimposed families of whole-cell K⁺ currents elicited by 450 ms test pulses from -80 to +80 mV in 10-mV steps. Lower graphs show the I-V relationship of the late current measured at the end of the test pulse and shows the mean \pm SEM at each potential. (A) Glucose (20 mM) reversibly inhibited the potassium currents of MIN6 and Huh7ins cells (left and centre columns; n = 4), however glucose did not affect the non-selective currents of Huh7 cells (right column; n = 3). (B) The channel opener diazoxide (100 μ M) reversibly increased the potassium currents of MIN6 and Huh7ins cells (left and centre columns; n = 9), but did not effect the non-selective currents of Huh7 cells (right column; n = 3).

To determine if functional K_{ATP} channels were present in Huh7ins or Huh7 cells, K_{ATP} channel currents were recorded using whole-cell patch-clamp electrophysiology, with MIN6 cells

being included as the positive control. Whole-cell patch-clamp recordings from potassium channels were made as previously described [27]. Cells grown on coverslips were transferred to a recording chamber and were perfused with a bath solution of the following composition (in mM): 140 Na acetate, 1 CaCl_2 , 1 MgCl_2 , 10 HEPES (pH 7.4). Patch pipettes were filled with an internal solution containing (in mM): 136 K acetate, 5 CsF, 5 KCl, 1 EGTA, 10 HEPES (pH 7.3). For inside-out patch-clamp recordings, the patch pipette was filled with (in mM): 135 NaCl, 5 KCl, 5 CaCl_2 , 2 MgSO_4 , 5 HEPES or a high K^+ extracellular solution in which KCl replaced NaCl. The bath solution contained (in mM): 107 KCl, 11 EGTA, 2 MgSO_4 , 1 CaCl_2 , 11 HEPES (pH 7.2). For Ca_v channel analyses the bath solution contained (in mM): 115 NaCl, 5 KCl, 10 CaCl_2 , 10 HEPES, 2 D-glucose and 100 μM tetrodotoxin (pH 7.4) and the internal solution contained (in mM): 10 CsCl, 115 Cs aspartate, 2.5 EGTA, 10 HEPES (pH 7.2). Channel currents were amplified and filtered using a MultiClamp amplifier (Molecular Devices, MDS Analytical Technologies, Toronto, Canada) and sampled on-line using a Digidata 1322 (A/D converter) and pClamp 8.2 software program (Molecular Devices).

The electrophysiological properties of the K_{ATP} channel in the Huh7ins cells closely resemble those reported for normal pancreatic β -cells [19]. The outward potassium currents of MIN6 and Huh7ins cells were sensitive to glucose and inhibited by perfusing 20 mM glucose for 5 min, with partial recovery of current amplitude after the washout of glucose for 10 min. In contrast, the non-selective outward and inward currents of Huh7 cells were not altered by the addition of 20 mM glucose (Figure 1A). The outward potassium currents of MIN6 and Huh7ins cells were also reversibly increased by perfusing with the K_{ATP} channel opener, diazoxide, 100 μM (Figure 1B), whereas the non-selective currents of Huh7 cells were unaffected by diazoxide.

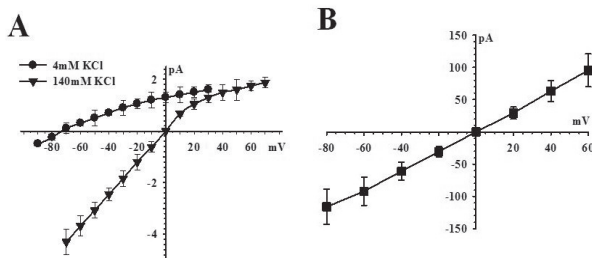


Figure 2. I-V curves of Huh7ins and Huh7 cells. (A) Mean current-voltage relations for inside-out patches of Huh7ins cells exposed to an external K^+ concentration of either 140 mM or 5 mM K^+ ($n = 6$). (B) Using an internal and external K^+ concentration of 5 mM and 140 mM respectively, the reversal potential (E_{rev}) of Huh7 whole-cell currents ($n = 6$), was approximately 0 mV, indicating a non-selective current. Values represent means \pm SEM.

Further support for the presence of functional K_{ATP} channels in Huh7ins cells was obtained by analysis of current-voltage (*I-V*) relationships of single channel currents, which had similar kinetics to that of pancreatic β -cells. Recordings were made from inside-out patches exposed to 140 mM $[\text{K}^+]_i$ and either 140 mM K^+ $[\text{K}^+]_o$ or 5 mM K^+ $[\text{K}^+]_o$. As would be expected

for a K^+ -selective channel, the single channel currents recorded with symmetrical $[K^+]_o$ reversed close to 0 mV, with a mean slope conductance of 48.5 pS (–80 to –10 mV). In comparison, the slope conductance was reduced to 12.4 pS (0 to +60 mV) when the $[K^+]_o$ was reduced to 5 mM, indicating that the channel was K^+ -selective (Figure 2A). In contrast the *I-V* curve for K_{ATP} channels in Huh7 cells indicated that currents from these cells were non-selective as the reversal potential was closer to 0 mV (Figure 2B). As secretory granules require K_{ATP} channels for the appropriate release of insulin [35, 36], it is likely that Huh7ins cells also contained K_{ATP} channels located intracellularly at the secretory granule membrane.

2.2. Secretion of insulin observed in real time in response to glucose and K_{ATP} channel blockers

In order to observe, in real time, the secretion of insulin from granules in response to stimulators and inhibitors of insulin secretion by confocal microscopy, Huh7 and MIN 6 cells were engineered to express insulin fused to EGFP. To accomplish this, human insulin cDNA pC₂ (a gift from Dr. M. Walker, Weizmann Institute, Israel) [7] was cloned into the multicloning site of the pEGFP-N1 vector (Clontech, CA, USA). As there were no intervening stop codons, EGFP/insulin (EGFPins) was expressed as a fusion protein, which allowed visualization and localization of the fusion protein in cells. The construct (20 µg) or vector alone was introduced into Huh7 and MIN6 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the instructions of the manufacturer. To obtain stable transfectants, containing the construct (EGFPins) or empty vector (EGFP), G418 antibiotic (0.55 mg/ml) (Gibco Laboratories, Grand Island, NY) was added to the culture medium after 48 h. Media and G418 were changed every 2–3 days. After 3–4 weeks of selection, 25 colonies were chosen and screened for production of insulin by radioimmunoassay (RIA) [7] and EGFP by fluorescence microscopy. Human c-peptide was measured as previously described [8]. Clones were expanded into mass cultures and maintained in G418 selection media (37°C, 5% CO₂). Huh7-EGFP (parental human liver cell line expressing EGFP) and Huh7-EGFPins (parental human liver cell line expressing EGFPins) cells were maintained in DMEM supplemented with 10% v/v fetal calf serum (FCS) (Trace Biosciences, Australia) in 5% CO₂ at 37°C. MIN6-EGFP (EGFP-expressing MIN6 cells) and MIN6-EGFPins (EGFPins-expressing MIN6 cells) cells were grown in DMEM supplemented with 15% v/v FCS (37°C, 5% CO₂). For these transfected cell lines, the selective antibiotic G418 (0.55 mg/ml) was added.

To compare the function of Huh7-EGFPins and Huh7ins cells, chronic insulin secretion, insulin storage, and glucose-responsiveness were assessed. Acute insulin secretion was measured by static stimulation in basal medium consisting of PBS supplemented with (in mM): 1 CaCl₂, 20 HEPES, 2 mg/ml BSA, 1.0 D-glucose; pH 7.4, as previously described [7]. Insulin was measured by RIA using human or rodent standards as previously described [7]. To assay insulin content, insulin was extracted from cells using 0.18 N HCl in 70% ethanol for 18 h at 4°C, as previously described [7]. To assess the quantity of human as compared to rodent insulin secreted by MIN6-EGFPins cells, a commercial RIA for human insulin (Linco Re-

search, MO, USA), was used. This has less than 1% and 6% cross-reactivity with rodent insulin and human proinsulin, respectively.

Of the 25 clones of Huh7-EGFPins isolated for analysis, insulin secretion differed 3-fold (0.11 ± 0.2 vs. 0.32 ± 0.2 pmol insulin/ 10^6 cells/24 h; $n = 6$) and insulin storage varied 2-fold (3.4 ± 1.2 vs. 7.1 ± 0.3 pmol insulin/ 10^6 cells; $n = 6$). Subsequently, six clones which secreted and stored the highest levels of insulin and exhibited consistently bright EGFP fluorescence, were examined for glucose responsiveness. Whilst all clones were glucose responsive, one clone (clone 16) was most comparable to Huh7ins cells [7] as it secreted equal amounts of insulin over a 24 h period (0.32 ± 0.2 vs. 0.30 ± 0.1 pmol insulin/ 10^6 cells for Huh7ins cells; $n = 6$). Insulin storage was also comparable between the two cell lines with Huh7-EGFPins (clone 16) and Huh7ins cells storing 7.1 ± 0.3 and 7.0 ± 0.2 pmol/ 10^6 cells ($n = 6$), respectively. Glucose concentration-response curves for the Huh7-EGFPins (clone 16) and Huh7ins cell lines were also determined and revealed that there was no significant difference to previously published values [7] (data not shown). Levels of human proinsulin (not insulin) were $11.4 \pm 1.2\%$ of total insulin ($n = 6$). Human c-peptide levels were $1.0 \pm 0.4\%$ of total insulin activity ($n = 6$). Therefore clone 16 was used for all subsequent analyses, and is referred to as Huh7-EGFPins hereafter. As expected, Huh7-EGFP cells did not synthesize, store nor secrete insulin. Examination of the insulin secreted chronically by MIN6-EGFPins cells revealed that $20.5 \pm 2.3\%$ ($n = 6$) was of human origin, the remainder being rodent insulin. Of the insulin stored by MIN6-EGFPins cells, $17.9 \pm 2.4\%$ ($n = 6$) was human insulin. As expected, all of the insulin stored and secreted by MIN6-EGFP cells was of rodent origin. These data suggest that MIN6 cells handled EGFPins in a similar fashion to native rodent insulin.

In order to perform confocal microscopy, cells were plated on coverslips (Marienfeld superior 22 mm diameter) and grown for 2–4 days. Each coverslip was inserted into a Perspex cell chamber, sealed with silicone grease, and overlaid with 1 ml DMEM containing 5 mM glucose (confocal scanning laser microscope [CSLM] medium). For Zinquin-E (zinquin ester, ethyl[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate) staining, cells were incubated at 37°C for 30 min with CSLM medium containing 25 μ M zinquin E (Luminis Pty Ltd, Australia), as previously described [27]. After incubation, cells were rinsed with CSLM medium before recording confocal images with a Leica TCSNT (Wetzlar, Germany) with an inverted microscope (Leica DMRBE). Cells were imaged with a UV laser, oil 100 \times (N.A.1.4 UV-corrected Planapo) or oil 63 \times (N.A.1.32 UV-corrected Planapo). Emissions were collected with a BP490/440 filter. For analyses of stable transfectants expressing EGFP, incubation with a fluorescent probe was not required. These cells were imaged with an Ar/Kr laser and DP488/568 dichroic and emissions were collected with a BP525/550 filter.

CSLM medium or test solutions containing glibenclamide (20 μ M), or diazoxide (150 μ M) in CSLM medium, or DMEM containing 20 mM glucose, were exchanged at 37°C. Density measurements on images were performed using the public domain NIH Image program [37].

Defined regions of interest (ROI) for individual cells (10–30 cells per experiment) were followed through a time series before, and after, addition of test solutions. All values were normalized by subtracting the initial density, before addition of the test solution, from all the measurements in the series for each individual ROI to give a value of zero density for the

initial time point. Confocal microscopy detected intracellular EGFP-ins or Zinquin-E as punctuate fluorescence, which was indicative of insulin stored within secretion granules.

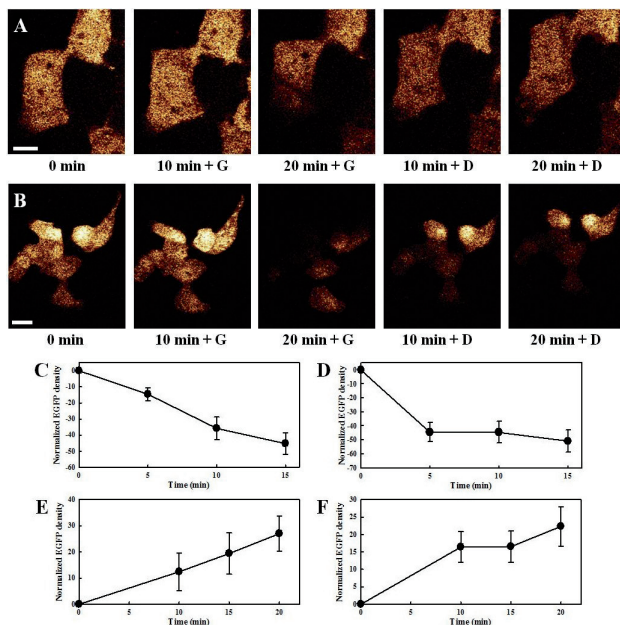


Figure 3. Confocal microscopic visualization of Huh7-EGFPins cells after exposure to glucose and diazoxide. (A) Huh7-EGFPins and (B) MIN6-EGFPins cells were incubated in DMEM containing 5 mM glucose (CLSM medium), then stimulated with glucose (20 mM, G) and diazoxide (150 μ M, D). Images were recorded in CLSM medium at 0, 10 and 20 min after glucose addition. At 20 min, cells were placed in CLSM medium containing diazoxide and images were recorded at 10 and 20 min after diazoxide exposure (bars = 10 μ m). Normalized EGFP density indicated that (C) Huh7-EGFPins ($n = 60$) and (D) MIN6-EGFPins cells ($n = 42$) showed decreasing EGFP density after addition of glucose, whereas (E) Huh7-EGFP ($n = 19$) and (F) MIN6-EGFP cells ($n = 12$) showed increasing EGFP density after addition of glucose. Values represent the mean \pm SEM.

For statistical analysis of all the confocal measurements described below SPSS version 11.5 (SPSS Inc) was used to determine a one-way analysis of variance after testing for homogeneity of variance using the Levene statistic. Huh7-EGFPins and MIN6-EGFPins cells responded in the same way to 20 mM glucose after 10 and 15 min, with loss of fluorescence from the ROI indicative of insulin secretion (Figure 3A-D). There was no significant difference between the response of Huh7-EGFPins and MIN6-EGFPins cells at 10 min ($p > 0.5$, $n = 60$) and 15 min ($p \geq 0.7$, $n = 42$). The MIN6-EGFPins cells responded more rapidly to the glucose stimulus than the Huh7ins-EGFP cells, with close to maximum loss of fluorescence achieved at 5 min, but after 10 min the two cell lines had achieved the same response level (Figure 3C-D). When diazoxide (150 μ M) was added to cells that had been stimulated by 20 mM glucose for 20 min, cytoplasmic fluorescence accumulated, as the release of insulin from

secretory granules was blocked (Figure 3A-B). The response of the control cell lines, Huh7-EGFP and Min6-EGFP to 20 mM glucose was significantly different ($p < 0.00001$) at all time points from that of the engineered cell lines. In the control cell lines fluorescence increased over time (Figure 3E-F) since cells accumulated considerable amounts of EGFP within their cytoplasm and they were unresponsive to 20 mM glucose. Presumably this phenomenon is attributable to an inability of the parental cell lines to direct EGFP to secretory granules, whereas the cells engineered to synthesize insulin responded by releasing insulin from secretory granules so that their fluorescence decreased (Figure 3C-D).

Huh7ins and Min6 cells stained with the zinquin-E probe responded to 20 mM glucose with decreasing fluorescence. There was no significant difference between Huh7ins cells labelled with zinquin-E and Huh7-EGFPins cells in their response to 20 mM glucose after 5 min ($p > 0.8$, $n = 41$) and 15 min ($P > 0.1$, $n = 59$). After incubation with the K_{ATP} channel blocking sulphonylurea, glibenclamide (20 μ M), the two cell lines responded as they did in the presence of glucose (i.e. decreasing fluorescence was observed). Conversely, treatment with 150 μ M diazoxide, which inhibits glucose-activated β -cell depolarisation by suppressing closure of K_{ATP} channels, caused increased fluorescence, showing that secretion of insulin was blocked in Huh7ins-EGFP cells (Figure 3A) and Huh7ins cells (with zinquin-E probe).

Huh7ins-EGFP and MIN6ins-EGFP cells responded to either glucose or glibenclamide with a decrease in fluorescence (indicative of insulin secretion). The same secretory response to glucose or glibenclamide was seen in MIN6 and Huh7ins cells using the zinquin probe. Through its high affinity for the sulphonylurea subunit of the K_{ATP} channel, glibenclamide renders the K_{ATP} channel inactive and calcium influx through Ca_v channels ensues due to depolarisation of the cell membrane. The release of insulin from intracellular storage granules is the net result of these processes. As this response to glibenclamide was observed for Huh7ins, Huh7-EGFPins, MIN6 and MIN6-EGFPins cells, insulin secretion likely occurred via the classic insulin triggering pathway utilized by pancreatic β -cells. In contrast, the negative controls (Huh7-EGFP and MIN6-EGFP cells) or Huh7 cells in the case of Zinquin-E labelling, were unresponsive to glucose or glibenclamide. The increased fluorescence of the negative control cells MIN6-EGFP and Huh7-EGFP after glucose stimulation showed that there was no trafficking of EGFP to secretory granules. In an earlier publication, Arvan and Halban [38] questioned the specificity of the trans Golgi network sorting process, but the fact that in our cell lines the secretion of EGFP-ins was regulated while EGFP was not, shows that the sorting of EGFP was specific, with only EGFP-insulin being trafficked to secretory granules.

2.3. Huh7ins cells express the K_{ATP} channel subunits, $K_{IR}6.2$, SUR2A and SUR2B, and the $\alpha 1$ -subunit of the $Ca_v1.3$ channel

Primers were designed to the cDNA sequences encoding the human K_{ATP} subunits, $K_{IR}6.2$ (F: AGCCCAAGTTCAGCATCTCTCC, R: CCAGAAATAGCATAGTGACAAGTGCC), SUR1 (F: TCAGGGTTGTGAACCGCA, R: GTTCTGCGAAGCATAGGC), SUR2A (F:

GGCAGGTGGGAAATCATCGTTA, R: TCCCCACCTTCAGTGACAA') and SUR2B (F: GATGCGGTTGCTACTGAA, R: ACTCCTTCACATGTCTGC). Primers were also designed to amplify the $\alpha 1$ -subunit of the $\text{Ca}_v1.3$ channel of pancreatic β -cells (F: TGGCAGGAGATCATGCTGG, R: CTAATCTCTTGCTCGCTACC). RT-PCR analyses were performed using the cDNA synthesised from RNA isolated from Huh7 and Huh7ins cells using TRIzol® Reagent (Invitrogen). Positive controls were HEPG2 cells that express the human $\text{K}_{\text{IR}}6.2$ and SUR2A subunits [27], or human pancreatic islets.

Immunoblot analyses were performed using protein extracted from Huh7 and Huh7ins cells and human pancreatic islets to detect the human K_{ATP} subunits, SUR1, SUR2A and SUR2B, and the $\alpha 1$ -subunit of the $\text{Ca}_v1.3$ channel. Detection of the $\text{K}_{\text{IR}}6.2$ subunit was determined as previously described [27]. For detection of $\text{K}_{\text{IR}}6.2$, SUR1, SUR2A, SUR2B and the $\alpha 1$ -subunit of the $\text{Ca}_v1.3$ channel, cell supernatants were suspended in buffer I containing (in mM): 10 Tris, 20 NaH_2PO_4 , 1 EDTA, 0.1 PMSF, 10 $\mu\text{g/ml}$ pepstatin, 10 $\mu\text{l/ml}$ leupeptin (pH 7.8), subjected to three freeze-thaw cycles, and then incubated for 20 min at 4°C. The protein concentration of the supernatant was determined using a Micro Bicinchoninic Protein Assay Reagent Kit (PIERCE, Thermo Fisher Scientific, Rockford, IL, USA). Protein samples (15 μg) were electrophoresed in 10% polyacrylamide gels (100 V) and then transferred to nitrocellulose membranes (Millipore Corporation, USA) for immunoblot analyses. Nitrocellulose membranes were blocked in PBS with 5% w/v skim milk overnight at 4°C. Immunoblotting was performed using a 1:1000 dilution of goat anti-human $\text{K}_{\text{IR}}6.2$, SUR1, SUR2A, SUR2B and the $\alpha 1$ -subunit of the $\text{Ca}_v1.3$ channel polyclonal IgGs (Santa Cruz Biotech. USA) and detection was achieved using monoclonal (mouse) anti-goat/sheep horseradish peroxidase IgG conjugate (1:800 dilution) (Sigma).

RT-PCR analysis revealed that the Huh7 and Huh7ins cells expressed the human K_{ATP} channel subunit, $\text{K}_{\text{IR}}6.2$, and the β -cell sulfonylurea receptor subunits, SUR2A and SUR2B (Figure 4A-C), together with the human $\alpha 1$ -subunit of the $\text{Ca}_v1.3$ channel (Figure 4E). SUR1 was only detected in the Huh7ins liver cell line (Figure 4D). Immunoblot analysis for the presence of $\text{K}_{\text{IR}}6.2$, SUR1, SUR2A and SUR2B, revealed strong expression in Huh7ins cells and human pancreatic islets, with no detectable expression in Huh7 cells (Figure 4F-I). The presence of protein product for the $\alpha 1$ -subunit of the $\text{Ca}_v1.3$ channel was confirmed by immunoblot analysis of protein extracted from Huh7ins cells, with only low expression in Huh7 cells (Figure 4J).

Thus, unlike the glucose-responsive insulin-secreting cell line, HEPG2ins/g [26], the Huh7ins cells expressed the SUR1 receptor as do pancreatic β -cells. The functional recording of K_{ATP} activity in Huh7ins cells are supported by the immunoblot analyses, which suggests that $\text{K}_{\text{IR}}6.2$, SUR1, SUR2A and SUR2B are strongly expressed in Huh7ins cells. There was no detectable expression of $\text{K}_{\text{IR}}6.2$, SUR1, SUR2A and SUR2B in Huh7 cells, which is supported by the absence of K_{ATP} currents in the patch-clamp recordings (Figure 2B). Expression of $\text{K}_{\text{IR}}6.2$ and SUR1, the two relevant subunits of the pancreatic β -cell K_{ATP} channel, is commonly seen in primary hepatocytes, although dedifferentiated cell lines such as HEPG2 [28] and Huh7 cells appear to have lost expression of SUR1 at the mRNA level. It is apparent that the process of pancreatic transdifferentiation, which has caused the formation

of secretory granules, has resulted in expression of $K_{IR}6.2$ protein and SUR1 at the mRNA level and protein expression in Huh7ins cells.

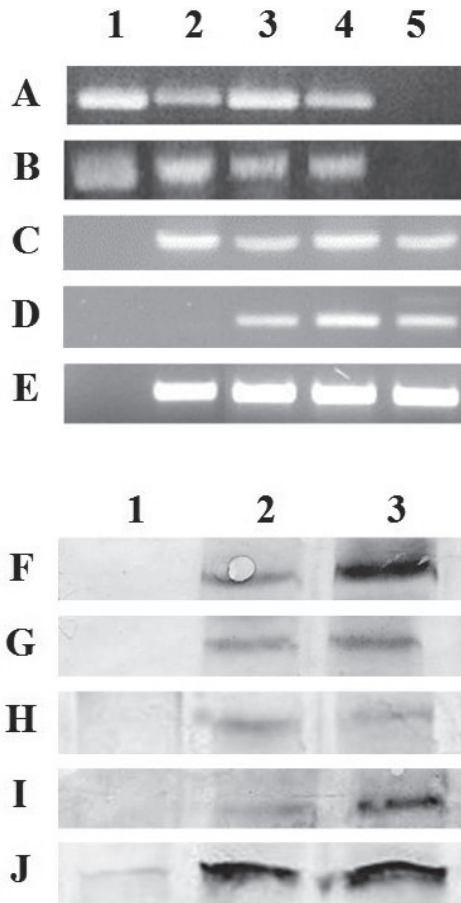


Figure 4. RT-PCR and immunoblot analysis for K_{ATP} and Ca_v channel subunits. RT-PCR analysis of liver cell lines for (A) human $K_{IR}6.2$: Huh7 (lane 1), Huh7ins (lane 2), Huh7-EGFPins (lane 3), HEPG2 (lane 4, positive control), and no cDNA control (lane 5); (B) human SUR2A: Huh7 (lane 1), Huh7ins (lane 2), Huh7-EGFPins (lane 3), HEPG2 (lane 4, positive control), and no cDNA control (lane 5); (C) human SUR2B: no cDNA (lane 1), human pancreas (lane 2, positive control), Huh7 (lane 3), Huh7ins (lane 4), Huh7-EGFPins (lane 5); (D) Human SUR1: no cDNA (lane 1), Huh7 (lane 2), Huh7ins (lane 3), Huh7-EGFPins (lane 4), human pancreas (lane 5, positive control), (E) human $\alpha 1$ -subunit of the $Ca_v1.3$ channel: no cDNA (lane 1), human pancreas (lane 2, positive control), Huh7 (lane 3), Huh7ins (lane 4), Huh7-EGFPins (lane 5). Immunoblot analysis for (F) human $K_{IR}6.2$, (G) human SUR2A, (H) SUR2B, (I) SUR1 and (J) the $\alpha 1$ -subunit of the $Ca_v1.3$ channel in Huh7 (lane 1), Huh7ins (lane 2) and human islet (lane 3).

2.4. Huh7ins cells possess Ca_v channels

The level of intracellular free Ca^{2+} was measured using Fluo4-AM and pluronic F-127 with a Zeiss microscope (Axiovert 200M; Zeiss, Germany). Cells were grown on coverslips until 50–70% confluent and were then incubated in culture medium containing 8 μM Fluo4-AM (Invitrogen, Carlsbad, CA) and 0.1% pluronic F-127 (Invitrogen) at 37°C for 60 min. To remove excess Fluo4-AM and F-127, the cells were incubated with HEPES buffer containing (in mM): 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 D-glucose, 10 HEPES (pH 7.4), for 30 min images were captured. The coverslips were then placed in a chamber containing HEPES buffer. After control images were taken (before addition of glucose or glibenclamide), the cells were exposed to 20 mM glucose or 20 μM glibenclamide until the completion of experiments. For the experiments in the presence of Ca_v channel blocker, the cells were incubated with 10 μM verapamil for 30 min before the addition of glucose or glibenclamide. Fluorescence intensity was observed under a Zeiss microscope and images were captured with a digital camera (AxioCam, Zeiss) and the Axiovision program (Zeiss). Images were taken every 20 s and analyzed using ImageJ software [39]. Results were presented as relative fluorescence values (F/F_0), where F_0 represents the fluorescence of controls (before addition of glucose or glibenclamide).

While the expression of Ca_v channels in pancreatic β -cells has been well documented [23, 40], their precise role in hepatocytes is yet to be elucidated. It has been reported that Ca_v1 channels are found in endocrine (pancreatic), cardiac and neural cells [41], but no physiologically-active Ca_v1 channels have been identified in hepatocytes prior to this study. Calcium imaging revealed that an increase in the extracellular glucose concentration from 5 to 20 mM immediately stimulated an elevated level of free $[\text{Ca}^{2+}]_i$ in Huh7ins cells, which peaked within 2 min and then gradually recovered to the level observed prior to application of 20 mM glucose (Figure 5A). The F/F_0 value at 2 min after the application of 20 mM glucose in Huh7ins cells was 1.14 ± 0.038 ($n = 33$, Figure 5B). However, 20 mM glucose did not significantly increase the level of free $[\text{Ca}^{2+}]_i$ in Huh7 cells ($F/F_0 = 1.02 \pm 0.01$, $n = 19$), which was significantly lower than that of Huh7ins cells (Fig 5A-B). To examine if blockade of K_{ATP} channels mimicked the effect of 20 mM glucose, glibenclamide (20 μM) was applied in the bath solution containing 5 mM glucose. Glibenclamide dramatically increased the level of intracellular free Ca^{2+} ($F/F_0 = 1.87 \pm 0.24$, $n = 25$), which had a similar time course to that observed in the presence of 20 mM glucose, but with a greater peak amplitude. Similar to the effects of 20 mM glucose on Huh7 cells, glibenclamide did not alter calcium flux in Huh7 cells (Figure 5A-B). It should be noted that both 20 mM glucose and 20 μM glibenclamide produced a more delayed increase in the $[\text{Ca}^{2+}]_i$ in Huh7 cells in comparison with data recorded in Huh7ins cells (Figure 5A).

Verapamil (10 μM), a phenylalkylamine $\text{Ca}_v1.x$ channel blocker, inhibited the increase in $[\text{Ca}^{2+}]_i$ in Huh7ins cells produced by 20 mM glucose (1.04 ± 0.02 , $n = 31$) and glibenclamide (0.99 ± 0.02 , $n = 31$; Figure 5A and C). This indicated that the observed glucose-induced block and diazoxide-induced increase in free $[\text{Ca}^{2+}]_i$ was mediated by Ca_v channels. To further validate this interpretation, we used the whole-cell patch-clamp technique to measure the effect of increased glucose on membrane currents in Huh7ins and Huh7 cells. The resultant $I-V$ curve indicated that increasing the concentration of glucose from 2 to 20 mM resulted in ac-

tivation of an inwardly-rectifying current in Huh7ins cells (Figure 5D). This current was blocked by the addition of CsCl thereby lending further support to the premise that it was mediated via K^+ channels. No activation was seen when Huh7 cells were used in these experiments (results not shown). Ca_v channel currents recorded from Huh7ins cells were inhibited by verapamil (10 μ M), indicating that $Ca_v1.x$ channels were involved in the response (Figure 5E). This further corroborates the calcium imaging data described above.

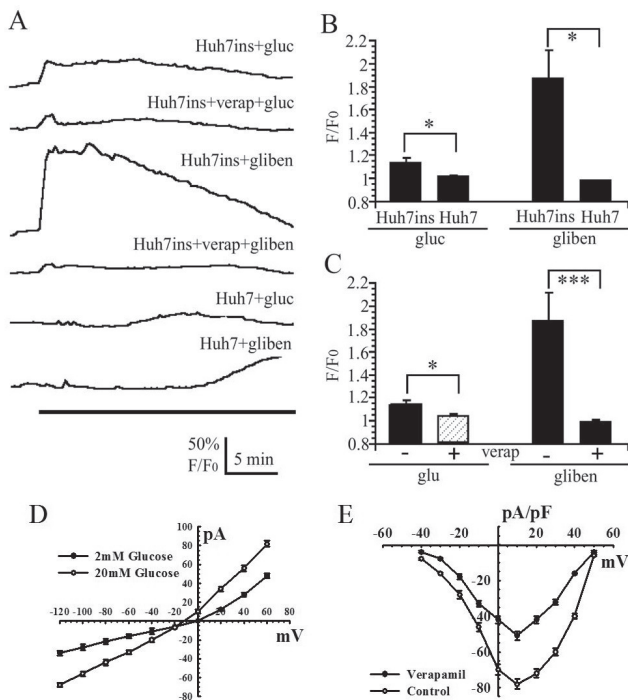


Figure 5. Calcium imaging and patch-clamp electrophysiology of Huh7ins and Huh7 cells. High glucose and blockade of K_{ATP} channels elevated levels of intracellular free Ca^{2+} in Huh7ins cells. (A) Averaged time courses of relative fluorescence intensity (F/F_0) induced by 20 mM glucose (gluc) and 20 μ M glibenclamide (gliben) in the presence, and absence, of 10 μ M verapamil (verap, $Ca_v1.x$ channel blocker) in Huh7ins and Huh7 cells. The black bar at the base of panel A represents the time of application of glucose or glibenclamide. Each trace represents an average F/F_0 value of the cells investigated. (B) Glucose and glibenclamide increased the level of free $[Ca^{2+}]_i$ in Huh7ins cells, but not in Huh7 cells. (C) Glucose- and glibenclamide-induced increases in intracellular free Ca^{2+} in Huh7ins cells were significantly inhibited by 10 μ M verapamil. The values shown in B and C were taken 2 min after application of glucose or glibenclamide. * $p < 0.05$ and *** $p < 0.001$. (D) Mean I-V relationship in Huh7ins cells under low (2 mM) and high (20 mM) glucose conditions ($n = 4$). (E) I-V curves for Ca_v channel currents in Huh7ins cells in the presence of 20 mM glucose and following the addition of 10 μ M verapamil ($n = 6$). Values are expressed as means \pm SEM

The $Ca_v1.3 \alpha 1$ subunit (Figure 4J), expressed in pancreatic β -cells [42], was detected in both Huh7ins cells and the parental Huh7 cells, at both the mRNA and the protein level, suggest-

ing that Huh7ins and Huh7 cells possess $\text{Ca}_v1.3$ channels that are similar to those found in pancreatic β -cells. Ca^{2+} imaging and patch-clamp electrophysiology experiments further detected a Ca_v channel current in Huh7ins cells, which was stimulated by glucose and inhibited by verapamil. The expression of functional Ca_v channels in Huh7ins cells may explain, in part, the acute secretion of insulin in response to glucose stimulation. The mechanism of insulin secretion depends upon the activities of ion channels in the plasma membrane, and, more critically, upon the activation of Ca_v channels, caused indirectly by increased glucose metabolism. Influx of Ca^{2+} , through open Ca_v channels, is responsible for the exocytosis of insulin storage granules, emphasising the importance of Ca_v channels in glucose-stimulated insulin secretion [41]. The lack of functional Ca_v channels in Huh7 cells is likely related to the low level of expression of the $\text{Ca}_v1.3$ α 1-subunit. Once it was determined that Huh7ins cells possessed functional Ca_v channels, static stimulation experiments using the inhibitor verapamil, and the activator BayK8644, established that Ca_v channels in Huh7ins cells function in a similar manner to Ca_v channels in pancreatic β -cells.

2.5. Huh7ins cells appear to be glucose-responsive through the presence of functional K_{ATP} channels and Ca_v channels

To measure insulin secretion, monolayers of cells were incubated with K_{ATP} channel modulators, using concentrations determined from concentration-response curves in the corresponding cell lines. These included the K_{ATP} channel activators tolbutamide (100 μM) or diazoxide (150 μM) and the K_{ATP} channel blocker glibenclamide (20 μM) with or without 20 mM glucose for 1 h. The effects of the Ca_v channel blocker verapamil (10 μM), the Ca_v channel activator Bay K8644 (1 μM), the sarcoplasmic and endoplasmic reticulum family of Ca^{2+} -ATPases (SERCA) blocker ryanodine (20 μM), the SERCA stimulator thapsigargin (1 μM), and the hemi-channel blocker oleic acid (20 μM) were also assessed. Inhibitors and activators were purchased from Sigma, Sydney, Australia. Results were expressed as means \pm standard error of the mean (SEM). The statistical analysis of insulin RIA results was by univariate repeated measures analysis of variance using Systat™ version 9. Post-hoc comparisons were made using Tukey's HSD test (Minitab™ version 13, Minitab Inc).

Stimulation with 20 mM glucose resulted in a 3.6- and 5.2-fold increase in insulin secretion by Huh7ins and MIN6 cells, respectively (Figures 6 and 7). Incubation of Huh7-EGFPins cells with the K_{ATP} channel blocker, glibenclamide, significantly increased insulin secretion by Huh7-EGFPins from 0.06 ± 0.01 to 0.26 ± 0.03 pmol/ 10^6 cells ($p < 0.001$, $n = 6$). The K_{ATP} activator, diazoxide, completely inhibited glucose-stimulated insulin release from Huh7-EGFPins (0.05 ± 0.02 pmol/ 10^6 cells, $n = 6$) and MIN6-EGFPins cells (data not shown). It was also noted that, diazoxide treatment prevented glucose-induced insulin secretion in Huh7ins and Huh7-EGFPins cells. Diazoxide causes sustained opening of K_{ATP} channels causing hyperpolarisation of the cell membrane, thereby preventing the voltage-dependant calcium response and inhibiting insulin exocytosis [43]. Static glucose stimulation experiments demonstrated that the insulin secretory response of Huh7ins and Huh7-EGFPins cells functioned via the channel-dependant pathway of insulin secretion. The responses of Huh7ins and MIN6 cells to diazoxide and

glibenclamide treatment were identical to that observed in each of the cell lines in which insulin was fused to EGFP (data not shown). Therefore, fusion of EGFP to insulin did not alter the physiological mechanism of insulin secretion.

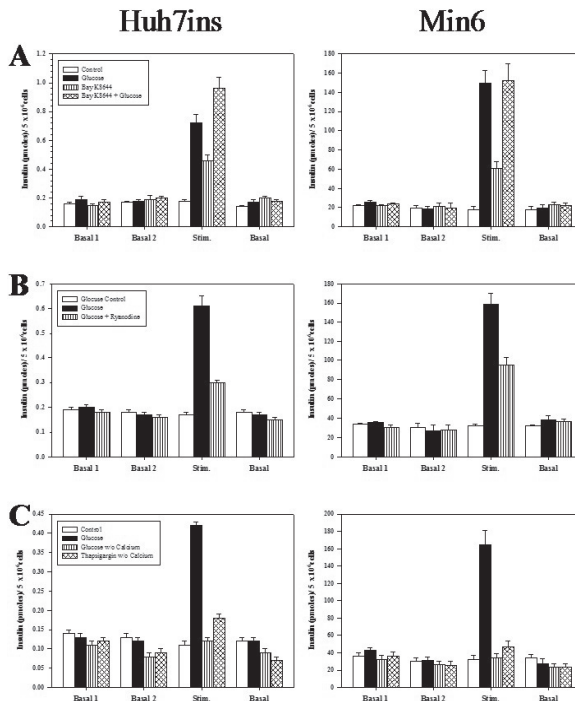


Figure 6. Secretion of insulin from Huh7ins cells and MIN6 cells. Insulin secretion was activated in response to 20 mM glucose alone or (A) 1 μ M BayK8644 \pm 20 mM glucose. (B) 20 μ M ryanodine \pm 20 mM glucose; and (C) 1 μ M thapsigargin in the absence of extracellular calcium. Cells were incubated in basal medium for two consecutive 1 h periods before being exposed to the stimulus for 1 h, followed by a third period of basal incubation. Cells in the control group were treated throughout with basal medium. Values are expressed as means \pm SEM ($n = 6$).

The application of the $\text{Ca}_v1.x$ channel activator, BayK8644 to Huh7ins and MIN6 cells significantly increased insulin secretion above basal levels ($p < 0.01$, $n = 6$; Figure 6A). In the presence of 20 mM glucose, BayK8644 further amplified glucose-stimulated insulin secretion in Huh7ins cells ($p < 0.05$, $n = 6$, Fig. 6A). Application of the SERCA blocker, ryanodine, which prevents increases in $[\text{Ca}^{2+}]_i$, caused a decrease in glucose-stimulated insulin secretion from Huh7ins and MIN6 cells ($p < 0.05$, $n = 6$; Figure 6B).

The dihydropyridine, BayK8644, functions as a Ca_v1 channel agonist, which interacts with the α_1 subunit of Ca_v channels to stabilise the channel in the open state, thereby enhancing Ca^{2+} influx to cause the exocytosis of insulin [41]. BayK8644 does not change the membrane

potential of resting β -cells [43]. Rather, it acts on the Ca_v channel in the open state, failing to affect basal insulin secretion at non-stimulatory glucose concentrations [43], but exaggerating glucose-stimulated insulin secretion [44, 45]. The addition of BayK8644 increased insulin secretion by both the Huh7ins and MIN6 cells. However, the amount of insulin secreted in the presence of BayK8644 was lower than that released in response to 20 mM glucose alone. Putatively, this concentration of glucose may have stimulated the influx of extracellular Ca^{2+} , the release of $[\text{Ca}^{2+}]_i$ from intracellular stores and increased Ca^{2+} via other Ca^{2+} -related pathways to such an extent that the total increase of Ca^{2+} in the cell was higher in the presence of 20 mM glucose as compared to BayK8644 alone.

Consistent with reports that BayK8644 is known to stimulate the opening of Ca_v channels in pancreatic β -cells without altering the membrane potential [44], static stimulation of Huh7ins cells with 1 μM BayK8644 plus 20 mM glucose amplified glucose-stimulated insulin release. However, BayK8644 failed to amplify glucose-stimulated insulin secretion in MIN6 cells. This finding may be attributable to the ability of 20 mM glucose alone to cause the maximum threshold in the activation of insulin release in MIN6 cells, such that the addition of BayK8644 was unable to exert any additional stimulatory effects. Nevertheless, these results demonstrate that the insulin secretory response of the Huh7ins cells is dependent upon the activation of Ca_v channels, as is the case for pancreatic β -cells.

Static stimulation of Huh7ins cells with the highly specific SERCA blocker thapsigargin, which induces the release of Ca^{2+} from intracellular stores resulted in a significant increase (two-fold increase over basal levels), in insulin secretion in the absence of extracellular Ca^{2+} ($p < 0.05$, $n = 6$; Figure 6C). Consistent with results from Tuch *et al.* [7], the response of the Huh7ins cells to glucose was abolished when Ca^{2+} was removed from the basal medium before 20 mM glucose was added ($p > 0.05$ vs. control, $n = 6$; Figure 6C). MIN6 cells showed a similar response; namely, in the absence of extracellular Ca^{2+} the glucose-responsiveness was abolished ($p > 0.05$ vs. control, $n = 6$; Figure 6C), and the presence of 1 μM thapsigargin significantly increased insulin secretion 1.8-fold over basal levels ($P < 0.05$, $n = 6$; Figure 6C).

The connexon (hemi-channel blocker), oleic acid, significantly reduced acute insulin secretion by 1.4-fold ($p < 0.05$, $n = 6$; Figure 7A), while verapamil (10 μM) resulted in a significant decrease in insulin secretion to glucose in both cell lines ($p < 0.05$, $n = 6$; Figure 7B). However, the combination of verapamil and ryanodine did not exert an additive effect on insulin secretion, compared to treatment with verapamil alone ($p < 0.05$, $n = 6$; Fig. 7B). Nevertheless, a greater decrease in insulin secretion was observed after the addition of verapamil, ryanodine and oleic acid in both Huh7ins ($p < 0.05$, $n = 6$) and MIN6 cells ($p < 0.05$, $n = 6$; Figure 7C).

SERCA operate to restore diminished intracellular endoplasmic and sarcoplasmic reticulum Ca^{2+} stores, thereby decreasing cytoplasmic Ca^{2+} levels [46-50]. Thapsigargin is a highly selective inhibitor of SERCA. Stimulation of β -cells with glucose causes an initial, thapsigargin-inhibitable, drop in $[\text{Ca}^{2+}]_i$ that precedes the increase in $[\text{Ca}^{2+}]_i$ due to the pumping of Ca^{2+} into the endoplasmic reticulum [51, 52]. Blocking of SERCA by thapsigargin augments the glucose-induced $[\text{Ca}^{2+}]_i$ increase by activating a depolarising store-operated current, which then facilitates the opening of Ca_v channels [51, 53, 54]. Consistent with the results reported by Tuch *et al.* [7], in the absence of extracellular Ca^{2+} , the glucose responsiveness of

both Huh7ins and MIN6 cells in the absence of extracellular Ca^{2+} , was lost, while normal glucose responsiveness was seen when Ca^{2+} was present in the medium. However, thapsigargin, which raises cytosolic Ca^{2+} , stimulated insulin secretion by both Huh7ins and MIN6 cells in the absence of extracellular Ca^{2+} . This finding further supports the role of intracellular Ca^{2+} storage in insulin secretion in both pancreatic β -cells and in the insulin-secreting liver cell line, Huh7ins.

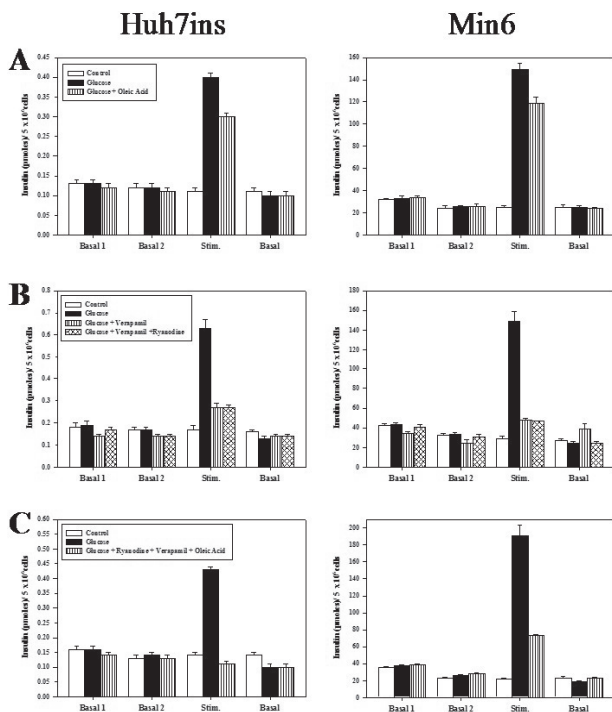


Figure 7. Secretion of insulin from Huh7ins cells and MIN6 cells. Insulin secretion was activated in response to 20 mM glucose alone or in the presence of (A) 20 μM oleic acid; (B) 10 μM verapamil \pm 10 μM ryanodine and (C) 10 μM verapamil, 20 μM ryanodine and 20 μM oleic acid. Cells were incubated in basal medium for two consecutive 1 h periods before being exposed to the stimulus for 1 h, followed by a third period of basal incubation. Cells in the control group were treated throughout with basal medium. Values are expressed as means \pm SEM ($n = 6$).

The presence of 20 μM ryanodine, which blocks Ca_v channels at concentrations $\geq 10 \mu\text{M}$ [55] and prevents the release of Ca^{2+} from the endoplasmic reticulum, reduced the glucose-responsiveness of both Huh7ins cells and MIN6 cells, although to a lesser extent than was observed in the presence of 10 μM verapamil. This finding is consistent with previous reports that intracellular Ca^{2+} stores (and therefore SERCA) contribute to the intracellular Ca^{2+} response during insulin secretion. The application of verapamil to Huh7ins cells caused a

complete abrogation of glucose-responsiveness upon extracellular Ca^{2+} levels has been previously reported for pancreatic β -cells [40, 43, 56]. As expected the addition of oleic acid to Huh7ins and MIN6 cells resulted in reduced glucose responsiveness, due to the blockage of hemi-channels, similar to what has been reported in pancreatic β -cells [57].

3. Conclusion

The results described in this chapter indicate that insulin secretion in engineered hepatocytes (Huh7ins cells) was controlled, as precisely as in the pancreatic β -cell, by a fully functional K_{ATP} and Ca_v channel system. The results clearly document that Huh7ins cells respond to glucose via insulin secretion from secretory granules by the same mechanism observed in pancreatic β -cells. This is the first study to demonstrate a clear physiological and biochemical interaction of K_{ATP} channels and Ca_v channels in liver cells, and as such reveals that hepatocytes are ideal candidates for the engineering of artificial β -cells. Testament to this, we have successfully engineered a liver cell line to synthesize, store and secrete insulin. Regardless of whether this hepatoma cell line will be a viable β -cell alternative for transplantation into patients, the present study provides valuable information with regards to the future engineering of glucose-responsive insulin-secreting liver cells. Elucidation of the minimal molecular modifications required for the creation of an artificial β -cell from a hepatocyte may one day provide therapeutic avenues to engineer a patient's own liver cells to synthesize, store and secrete insulin in response to metabolic stimuli.

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Feasibility of Gene Therapy for Tooth Regeneration by Stimulation of a Third Dentition

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Additional information is available at the end of the chapter

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1. Introduction

The tooth is a complex biological organ that consists of multiple tissues, including enamel, dentin, cementum, and pulp. Missing teeth is a common and frequently occurring problem in aging populations. To treat these defects, the current approach involves fixed or removable prostheses, autotransplantation, and dental implants. The exploration of new strategies for tooth replacement has become a hot topic. Using the foundations of experimental embryology, developmental and molecular biology, and the principles of biomimetics, tooth regeneration is becoming a realistic possibility. Several different methods have been proposed to achieve biological tooth replacement[1-8]. These include scaffold-based tooth regeneration, cell pellet engineering, chimeric tooth engineering, stimulation of the formation of a third dentition, and gene-manipulated tooth regeneration. The idea that a third dentition might be locally induced to replace missing teeth is an attractive concept[5,8,9]. This approach is generally presented in terms of adding molecules to induce *de novo* tooth initiation in the mouth. It might be combined with gene-manipulated tooth regeneration; that is, endogenous dental cells *in situ* can be activated or repressed by a gene-delivery technique to produce a tooth. Tooth development is the result of reciprocal and reiterative signaling between oral ectoderm-derived dental epithelium and cranial neural crest cell-derived dental mesenchyme under genetic control [10-12]. More than 200 genes are known to be expressed during tooth development (<http://bite-it.helsinki.fi/>). A number of mouse mutants are now starting to provide some insights into the mechanisms of supernumerary tooth formation. Multiple supernumerary teeth may have genetic components in their etiology and partially represent the third dentition in hu-

mans. Such candidate molecules or genes might be those that are involved in embryonic tooth induction, in successional tooth formation, or in the control of the number of teeth. This means that it may be possible to induce *de novo* tooth formation by the in situ repression or activation of a single candidate gene. In this review, we present an overview of the collective knowledge of tooth regeneration, especially regarding the control of the number of teeth for gene therapy by the stimulation of a third dentition.

2. The third dentition

It has been suggested that, in humans, a “third dentition” with one or more supernumerary teeth can occur in addition to the permanent dentition, and supernumerary teeth are sometimes thought to represent a partial post-permanent dentition [13-15]. The basic dentition pattern observed in mammals is diphyodont, and consists of three incisors, one canine, four premolars, and three molars, while Human teeth are diphyodont excepting the permanent molars [16]. The deciduous teeth are, ontogenetically, the first generation of teeth. The permanent teeth (except molar) belong to the second dentition. The term “third dentition” refers to the opinion that one more set of teeth can occur in addition to the permanent teeth (Figure 1). Human teeth are diphyodont excepting the permanent molars. The normal mouse dentition is monophyodont and composed of one incisor and three molars in each quadrant. The number of teeth is usually strictly determined. It was initially reported that there is an anlage of the third dentition in some mammals [17]. The presence of an epithelial anlage of the third dentition was also noticed in humans [18,19]. The teeth and anlagen that appear in third dentition in serial sections of infant jaws and some fetuses have been analyzed. The epithelium which is considered as the anlagen of the third dentition develops lingual to all permanent tooth germs [15]. Furthermore, when it appears, the predecessor (permanent tooth germ) is in the bell-shaped stage [15]. The timing of appearance of the third dentition seems to be after birth (Table 1). This means that we have a chance to access the formation of the third dentition in the mouth.

Teeth	The time of appearance of the third dentition	
	Maxilla	Mandible
central incisors	~ 3 months after birth	2 ~ 3 months after birth
lateral incisors	8 ~ 9 months after birth	2 ~ 3 months after birth
canines	2 ~ 7 months after birth	2 ~ 3 months after birth
the first premolar	1 year 1 month ~ 5 years 4 months after birth	1 year 1 month ~ 5 years 4 months after birth
the second premolar	1 year 1 month ~ 5 years 4 months after birth	2 years ~ 5 years 4 months after birth

Table 1. Timing of appearance of the third dentition

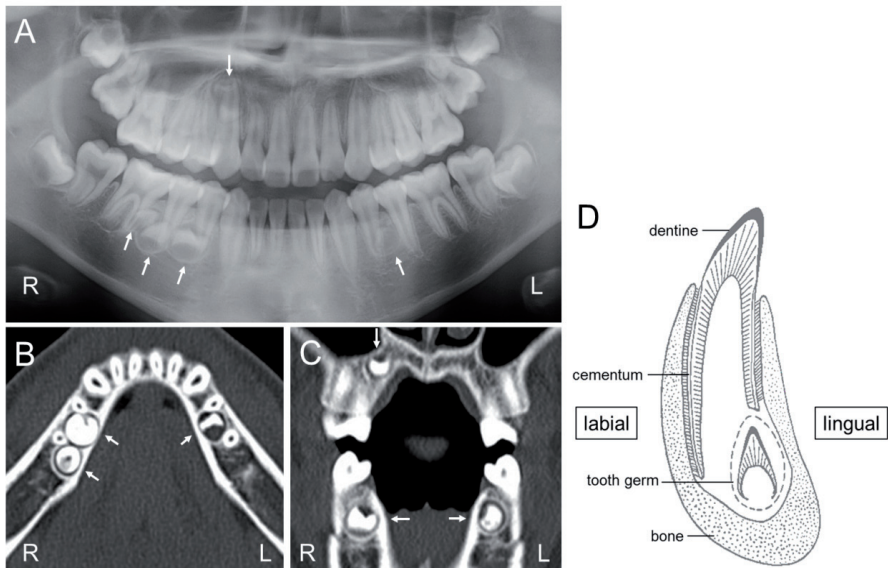


Figure 1. Multiple impacted supernumerary teeth in a 13-year-old non-syndromic patient. The third dentition develops lingual to the permanent tooth germ (D). All impacted supernumerary teeth in this patient are located to the lingual side of the permanent teeth (white arrow) (A-C). These multiple supernumerary teeth seem to be post-permanent dentition ("third dentition").

Analysis of other model systems with continuous tooth replacement or secondary tooth formation, such as in the fish, snake, lizard, and ferret, is providing insights into the molecular and cellular mechanisms underlying successional tooth development, and will assist in studies on supernumerary tooth formation in humans. While some nonmammalian species have multi rowed dentition and replace their teeth regularly throughout life, mammalian vertebrates have one row of teeth and only renew their teeth once, or, in some rodents, show no replacement [20-23]. Detailed histological analysis of the tooth replacement in these models indicates that the successional teeth are initiated from the dental lamina epithelium, which grows from the lingual side of the deciduous tooth enamel organ, and it later elongates and buds into the jaw mesenchyme, forming successional teeth. Jarvien et al. showed that, in the ferret, *Sostdc1* (also known as USAG-1, ectodin, and Wise) is expressed in the elongating successional dental lamina at the interface between the lamina and deciduous tooth, as well as the buccal side of the dental lamina, suggesting that *Sostdc1* plays a role in defining the identity of the dental lamina [20]. Handrigan et al. analyzed successional tooth formation in the snake and in lizard, and proposed that dental epithelium stem cells are responsible for the formation of successional lamina, and Wnt signaling may regulate the stem cell fate in these cells [24]. Maintenance or reactivation of component dental lamina is thus pivotal for the replacement tooth and supernumerary formation.

3. Human syndromes associated with supernumerary teeth

Supernumerary teeth can be associated with a syndrome (Table 2) or they can be found in non-syndromic patients [25-28]. Only 1% of non-syndromic cases have multiple supernumerary teeth, which occur most frequently in the mandibular premolar area, followed by the molar and anterior regions, respectively [29-34]. There are special cases exhibiting permanent supernumerary teeth developing as supplementary teeth forming after the permanent teeth. These are thought to represent a third dentition, best known as manifestations of cleidocranial dysplasia (CCD).

Syndrome	Gene	Genetics	References
Cleidocranial Dysplasia; CCD (Dental anomalies, isolated dental phenotype) (MIM 119600)	Runx2 (MIM 600211)	Chromosome 6p21, autosomal dominant	Lee et al., 1997; Mundlos et al., 1997.
Familial adenomatous polyposis 1; FAP1 (including Gardner syndrome) (MIM 175100)	APC (MIM 611731)	Chromosome 5q21-22, autosomal dominant	Fader et al., 1962; Ida et al., 1981; Shafer et al., 1983; Jensen and Kreiborg, 1990.
Nance–Horan syndrome (Cataract-Dental syndrome) (MIM 302350)	NHS (MIM 300457)	Chromosome Xp22.13, X-linked dominant	Bixler et al., 1984; Van Dorp and Delleman, 1979; Walpole et al., 1990; Burdon et al., 2003.
Trichorhinophalangeal syndrome, Type III (TRPS3) (MIM 190351)	TRPS1 (MIM 604386)	Chromosome 8q23.3, autosomal dominant	Giedion., 1966; Momeni et al., 2000; Kantaputra et al., 2008.
Robinow syndrome (MIM 180700)	WNT5A (MIM 164975)	Chromosome 3p14.3, autosomal dominant	Mazzeu et al., 2007.
Hallermann-Streiff syndrome; HSS (MIM 234100)	GJA1 (MIM 121014)	Chromosome 6q22.31, Isolated cases	da Fonseca and Mueller, 1994; Robotta and Schafer, 2011.
Rothmund–Thomson Syndrome; RTS (MIM 268400)	RECQL4 (MIM 603780)	Chromosome 8q24.3, autosomal recessive	Kitao et al., 1999.
Orofaciodigital Syndrome I ; OFD1 (Papillon-Leage and Psaupe syndrome) (MIM 311200)	OFD1 (MIM 300170)	Chromosome Xp22.2, X-linked dominant	Ferrante et al., 2001.
Uncombable Hair, Retinal Pigmentary Dystrophy, Dental Anomalies, and Brachydactyly (Bork Syndrome) (MIM 191482)	Unknown	autosomal dominant	Silengo et al., 1993.

Table 2. Human syndromes associated with supernumerary teeth

Genetic mutations have been associated with the presence or absence of individual types of teeth. Supernumerary teeth are associated with more than 20 syndromes and developmental abnormalities like CCD, and Gardner syndrome [35]. The percentage occurrence in CCD is 22% in the maxillary incisor region and 5% in the molar region[36-38]. CCD is a dominantly inherited skeletal dysplasia caused by mutations in *Runx2* [39-40]. It is characterized by persistently open sutures or the delayed closure of sutures, hypoplastic or aplastic clavicles, a short stature, delayed eruption of permanent dentition, supernumerary teeth, and other skeletal anomalies. There is a wide spectrum of phenotypic variability ranging from the full-blown phenotype to an isolated dental phenotype characterized by supernumerary tooth formation and/or the delayed eruption of permanent teeth in CCD (Figure 1) [41-44]. A dose-related effect seems to be present, as the milder case of CCD, and those exhibiting primary dental anomalies, are related to mutations that reduce, but do not abolish, protein stability, DNA binding, and transactivation [41,43-45]. *Runx2*-deficient mice were found to exhibit lingualbuds in front of the upper molars, and these were much more prominent than in wild-type mice[46,47]. These buds presumably represent the mouse secondary dentition, and it is likely that *Runx2* acts to prevent the formation of these buds. *Runx2* usually functions as a cell growth inhibitor[43]. *Runx2* reg-

ulates the proliferation of cells and may exert specific control on the dental lamina and formation of successive dentitions. Runx2 heterozygous mutant mice mostly phenocopied the skeletal defects of CCD in humans, but with no supernumerary tooth formation [48] (Otto, 1997). Notably, in Runx2 homozygous and heterozygous mouse upper molars, a prominent epithelial bud regularly presents. This epithelial bud protrudes lingually with active Shh signaling, and it may represent the extension of the dental lamina for successional tooth formation in mice. Hence, although Runx2 is required for primary tooth development, it prevents the growth of the dental lamina and successional tooth formation [47].

Familial adenomatous polyposis (FAP), also named adenomatous polyposis of the colon (APC), is an autosomal dominant hereditary disorder characterized by the development of many precancerous colorectal adenomatous polyps, some of which will inevitably develop into cancer. In addition to colorectal neoplasm, individuals can develop variable extracolonic lesions, including upper gastrointestinal polyposis, osteomas, congenital hypertrophy of the retinal pigment epithelium, soft tissue tumors, desmoid tumors, and dental anomalies [49-53]. Dental abnormalities include impacted teeth, congenital absence of one or more teeth, supernumerary teeth, dentigerous cysts associated with the crown of an unerupted tooth, and odontomas [50,52]. Gardner syndrome is a variant of FAP characterized by multiple adenomas of the colon and rectum typical of FAP together with osteomas and soft tissue tumors [49,51]. Supernumerary teeth and osteomas were originally described as a part of Gardner syndrome, but they can also occur in FAP patients with or without other extracolonic lesions [51,52]. FAP and Gardner syndrome are caused by a large number of germinal mutations in the *APC* gene [52,53]. *APC* is a tumor suppressor gene involved in the down-regulation of free intracellular β -catenin, the major signal transducer of the canonical Wnt signaling pathway, as well as a central component of the E-cadherin adhesion complex [54,55]. In addition, the *APC* protein may also play roles in chromosomal stability, the regulation of cell migration up the colonic crypt and cell adhesion through association with GSK3 β , and other functions associated with microtubule bundles [55,56]. Inactivation of *APC* would lead to the stabilization and accumulation of the proto-oncogene β -catenin, dysregulation of the cell cycle, and chromosomal instability [52]. Approximately 11-27% of patients have supernumerary teeth, but, so far, no specific codon mutation of the *APC* gene has been found to correlate with supernumerary teeth. Correlations seem to exist between dental abnormalities and the number and type of osteomas, with the highest incidence of supernumerary teeth and odontomas being found in FAP patients with three or more osteomas [52]. Conditional knockout of the *Apc*-gene resulted in supernumerary teeth in mice [57-59]. Notably, adult oral tissues, especially young adult tissues, are still responsive to the loss of *Apc* [60]. In old adult mice, supernumerary teeth can be induced on both labial and lingual sides of the incisors, which contain adult stem cells supporting the continuous growth of mouse incisors [60,61]. In young mice, supernumerary tooth germs were induced in multiple regions of the jaw in both incisor and molar regions. They can form directly from the oral epithelium, in the dental lamina connecting the developing molar or incisor tooth germ to the oral epithelium, in the crown region, as well as in the elongating and furcation area of the developing root [60].

The identification of mutations in *RUNX2* causing an isolated dental phenotype in CCD and in *APC* causing FAP has attracted attention as a possible route towards inducing *de novo* tooth formation.

4. Supernumerary tooth formation in a mouse model

The number of teeth is usually strictly determined. Whereas evidence supporting a genetic etiology for tooth agenesis is well established, the etiology of supernumerary tooth formation is only partially understood in the mouse model (Table 3). Unlike humans, mice have only molars and incisors separated by a toothless region called the diastema. In addition, mice only have a single primary dentition and their teeth are not replaced. Therefore, mice may not be an optimal model for studying tooth replacement and supernumerary tooth formation [62]. Most of the reported mouse supernumerary teeth are located in the diastema region. This is not a *de novo* tooth formation but the rescue of vestigial tooth rudiments. During the early stages of tooth development, many transient vestigial dental buds develop in the diastema area. Some of them can develop into the bud stage, but later regress and disappear by apoptosis, or merge with the mesial crown of the first molar tooth [63–68]. Major signaling pathways regulating tooth development are also expressed in these vestigial dental buds. Modulation of these signals can rescue these vestigial tooth rudiments to develop into supernumerary diastema teeth [23]. A number of mutant mouse strains have been reported exhibiting supernumerary diastema teeth. Although the rudimentary tooth buds form in the embryonic diastema, they regress apoptotically [69]. Transgenic mice in which the keratin 14 promoter directs Ectodysplasin (Eda), a member of the tumor necrosis factor (TNF) family of signaling molecules, or Eda receptor expression to the epithelium had supernumerary teeth mesial to the first molar as a result of diastema tooth development [70–72]. It has also been reported that Sprouty2 (Spry2) or Spry4 (which encode negative feedback regulators of fibroblast growth factor (FGF)) deficient mice showed supernumerary tooth formation as a result of diastema tooth development [73]. Hypomorphic Polaris mice and *Wnt-Cre* (Polaris conditional mutant mice with affected Shh signaling) [73–74], Pax6 mutant mice [75] and Gas1 null mutants [73] were also included. Uterine sensitization associated gene-1 (USAG-1) is a BMP antagonist, and also modulates Wnt signaling. We reported that USAG-1-deficient mice have supernumerary teeth (Figure 2).

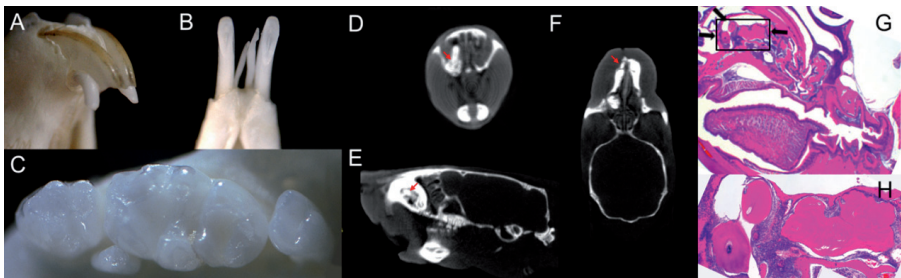


Figure 2. Supernumerary teeth formation in *Sostdc 1* (USAG-1) (A–C) and *CEBPB* (D–H) adult mutant mice. A: Oblique view of the maxillary incisors. B: Occlusal view of the mandibular incisors. C: Occlusal view of the mandibular molars. Micro-CT images (D–F) and HE-staining (G,H) of the murine head. A frontal view (D), a sagittal view (E) and a horizontal view (F) showed supernumerary tooth (red arrow). Two supernumerary teeth and an odontoma were seen in a low (G) and a high (H) magnification.

Mutant mouse	Tooth phenotype	references
Sostdc1 ^{-/-} (USAG-1, ectodin, Wise)	Supernumerary incisors in the maxilla and mandible Premolar mesial to first molar, peg-shaped tooth lingual to first molar	Munne et al., 2009; Ohazama et al., 2008; Yanagita et al., 2006; Murashima-Suginami et al., 2007, 2008.
CEBPB ^{-/-}	Supernumerary teeth and/or odontomas in the diastema between the incisor and the first molar	Huang et al., 2012.
Gas1 ^{-/-}	Premolar mesial to first molar, both jaws (100% penetrance)	Ohazama et al., 2009.
Gas1 ^{-/-} ; Shh ^{+/+}	Mandibular molar (associated with jaw duplication)	Seppala et al., 2007.
Tg737 ^{opk} hypomorph	Premolar mesial to first molar, both jaws (100% penetrance)	Zhang et al., 2003; Ohazama et al., 2009,.
Wnt1-Cre; Polaris ^{fllox}	Premolar mesial to first molar, both jaws (100% penetrance)	Ohazama et al., 2009.
Spry2 ^{-/-}	Premolar mesial to first molar; maxilla (>5%), mandible (97%:92% bilateral; 5% unilateral)	Klein et al., 2006; Peterkova et al., 2009.
Spry4 ^{-/-}	Both jaws? 16% penetrance (most unilateral)	Klein et al., 2006.
Lrp4 ^{-/-} (Megf7) hypomorph	Supernumerary incisors in the maxilla and mandible Premolar mesial to first molar (varying penetrance in both jaws) Lingual peg-shaped tooth (maxilla, variable penetrance)	Ohazama et al., 2008.
Osr2 ^{-/-}	Lingual molars	Zhang et al., 2009.
Epiprotein ^{-/-}	Multiple incisors and molars in both jaws	Nakamura et al., 2008.
K14-Cre; Apc ^{cko/cko}	Multiple incisor and molar tooth buds	Kuraguchi et al., 2006.
K14-Cre ^{8Bm} ; Apc ^{cko/cko}	Numerous labial and lingual incisor and molar teeth (↑ with age)	Wang et al., 2009.
K14-Cre ^{1Amc} ; Apc ^{cko/cko}	Numerous epithelial buds from E14.5	Wang et al., 2009.
K14-CreER TM ; Apc ^{cko/cko}	Numerous labial and lingual incisors (age P5–10/12)	Wang et al., 2009.
K14-CreER TM ; Ctnb1 ^{(ex3)/fl+}	Numerous labial and lingual incisors (age P5–6/12) P5 molar supernumeraries	Wang et al., 2009.
K14-Cre/+; β-catenin ^{ex3fl/+}	Multiple incisor and molar epithelial invaginations in both jaws	Järvinen et al., 2006.
K14-Cre; Ctnnb1 ^{(ex3)/fl+}	Multiple molar epithelial invaginations	Liu et al., 2008.
K14-Lef1	Rudimentary teeth at inappropriate sites	Zhou et al., 1995.
Pax6 ^{Sey}	Incisor supernumeraries: 35% unilateral; 45% bilateral incisors	Kaufman et al., 1995.
K14-Eda	Premolar mesial to first molar; incomplete penetrance	Kangas et al., 2004; Mustonen et al., 2003.
K14-Edar	Premolar mesial to first molar; incomplete penetrance	Tucker et al., 2004.
Tabby ^{+/+}	Molar (2.5%; mandible > maxilla)	Gruneberg et al., 1966; Sofaer et al., 1969.
B6CBACa-A ^{+/+} A-Eda ^{Ta/0}	Molar (1%; mandible >maxilla)	Peterkova et al., 2005.
di	Mandibular incisors (right > left)	Danforth et al., 1958.
β-cat ^{Δex3K14/+}	Supernumerary molars in the maxilla and mandible	Järvinen, E., 2006

Table 3. Mutant mouse associated with supernumerary teeth

The supernumerary maxillary incisor appears to form as a result of the successive development of the rudimentary upper incisor. USAG-1 abrogation rescued apoptotic elimination of odontogenic mesenchymal cells [14]. BMP signaling in the rudimentary maxillary incisor, assessed by expressions of *Msx1* and *Dlx2* and the phosphorylation of Smad protein, was significantly enhanced. Wnt signaling, as demonstrated by the nuclear localization of β-catenin, was also up-regulated. The inhibition of BMP signaling rescues supernumerary tooth formation in E15 incisor explant culture. Based upon these results, we conclude that enhanced BMP signaling results in supernumerary teeth and BMP signaling was modulated by Wnt signaling in the USAG-1-deficient mouse model (Figure 3) [76]. Canonical Wnt/β-catenin signaling and its down-stream molecule Lef-1 are essential for tooth development [77].

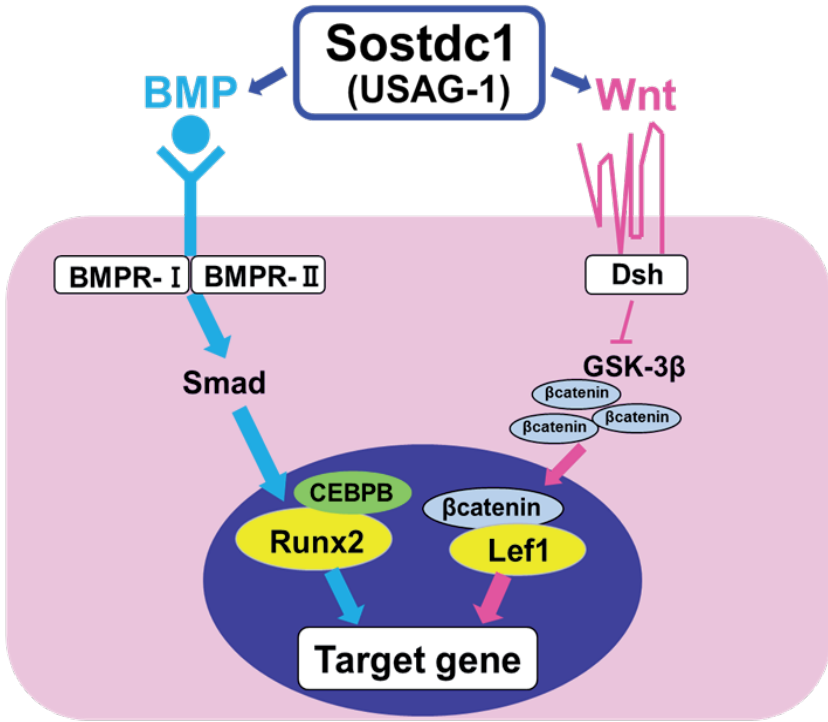


Figure 3. Diagrammatic representation of the Sostdc (USAG-1) pathway during development

Overexpression of Lef-1 under the control of the K14 promoter in transgenic mice leads to the development abnormal invaginations of the dental epithelium in the mesenchyme and formation of a tooth-like structure [78]. *De novo* supernumerary teeth arising directly from the primary tooth germ or dental lamina have been reported in *Apc* loss-of-function (as discussed in the previous section) or β -catenin gain-of-function mice, and *Sp6* (*Epiprofin*)-deficient mice. It was demonstrated that mouse tooth buds expressing stabilized β -catenin give rise to extra teeth[58] (Jarvinen et al., 2006). More recently, Epiprofin (Epf) (a zinc finger transcription factor belonging to the Sp transcription factor superfamily)-deficient mice developed an excess number of teeth[79]. Mammals only have one row of teeth in each jaw. Interestingly, in the *Osr2* null mutant mouse embryo, supernumerary tooth germs were found developing directly from the oral epithelium lingual to their molar tooth germs [80]. More recently, we also demonstrated that CEBPB deficiency was related to the formation of supernumerary teeth[81]. A total of 66.7% of CEBPB^{-/-} 12-month-olds sustained supernumerary teeth and/or odontomas in the diastema between the incisor and the first molar. Two supernumerary teeth accompanied with a complex odontoma near the root of the upper right incisor were identified in a CEBPB^{-/-} adult (Figure 2), whilst two other CEBPB^{-/-} mice simply

showed a supernumerary tooth in the upper left quadrant. Another CEBPB^{-/-} adult mouse did not display any supernumerary teeth in either jaw, but an odontoma in the lower-right quadrant. All of the CEBPB^{-/-} adults appeared with a normal number of erupted incisors and molars. Nevertheless, 20% of the CEBPB^{-/-} 12-month-olds had a missing lower third molar. Dental anomalies such as supernumerary teeth, odontomas, or hypodontia were not found in mice of any other genotypes and/or age[81].

These mouse models clearly demonstrated that it was possible to induce *de novo* tooth formation by the in situ repression or activation of single candidate gene such as USAG-1.

5. Gene therapy approaches

Gene therapy provides a unique tool for the delivery of previously identified signaling molecules in both time and space that may significantly augment our progress toward clinical tooth regeneration. Stimulation of the formation of a third dentition and gene-manipulated tooth regeneration comprise an attractive concept (Figure 4). This approach is generally presented in terms of adding molecules to induce *de novo* tooth initiation in the mouth. It might be combined with gene-manipulated tooth regeneration; that is, endogenous dental cells *in situ* can be activated or repressed by a gene-delivery technique to make a tooth. We have a chance to access the formation of the third dentition in the mouth, because the time of appearance of the third dentition seems to be after birth. As the half-life of targeted proteins *in vivo* is transient, tooth regeneration is not a common outcome following conventional therapy. Typically, high concentrations are required to promote regeneration [82]). Therefore, supplemental local production via gene transfer could be superior to bolus delivery methods.

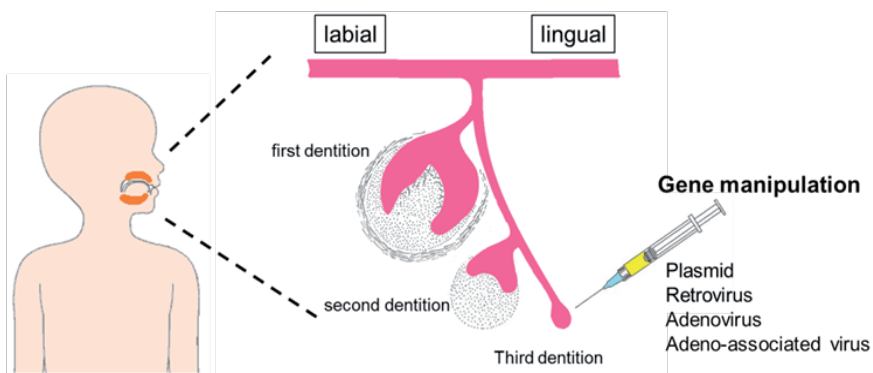


Figure 4. *In vivo* gene delivery approach for the tooth regeneration by stimulation of a third dentition.

Simply stated, gene therapy consists of the insertion of genes into an individual's cells directly or indirectly with a matrix to promote a specific biological effect. Gene therapy can be used to induce a more favorable host response. Targeting cells for gene therapy requires the use of vectors or direct delivery methods to transfect them. To overcome the short half-lives of peptides *in vivo*, gene therapy that uses a vector that encodes the candidate genes is utilized to stimulate the formation of the third dentition. The two main strategies of gene vector delivery have been applied. Gene vectors can be introduced directly to the target site (*in vivo* gene delivery) [83] or selected cells can be harvested, expanded, genetically transduced, and then reimplanted (*ex vivo* gene delivery). *In vivo* gene transfer involves the insertion of the gene of interest directly into the body, anticipating the genetic modification of the target cells. *Ex vivo* gene transfer includes the incorporation of genetic material into cells exposed from a tissue biopsy with subsequent reimplantation into the recipient. So far, *in vivo* gene delivery has been a suitable gene therapy approach in tooth regeneration by stimulation of the third dentition, but *ex vivo* gene delivery is not realistic because of the poor availability of ideal cells.

Gene transfer is accomplished through the use of viral and nonviral vectors. The three main classes of virus used for gene therapy are the retrovirus, adenovirus, and adenoassociated viruses. Retroviruses are ideal for long-term gene therapy since, once introduced, their DNA integrates and becomes part of the genome of the host cells. Indeed, the current human genome contains up to 5 to 8% of endogenous retroviral sequences that have been acquired over the course of evolution [84]. Adenoviruses are more commonly suited for short-term gene delivery and are highly targeted for tissue engineering strategies that desire protein production over the course of several weeks. Efficient adenovirus-directed gene delivery to odontogenic mesenchymal cells derived from cranial neural crest cells was reported [85,86]. In addition, because the adenovirus is well-known as the "virus of the common cold," infection is generally nontoxic and self-limiting. However, determination of the genotoxicity for each specific application is necessary to keep the safety profile within acceptable parameters. Adenoassociated viruses have become the focus of much research in recent years because of their complete inability to replicate without a helper virus, potential for tissue-specific targeting, and gene expression in the order of months to years. The ability to specifically target one tissue type without adverse effects on neighboring tissues is highly desired in fields such as tooth regeneration. On the other hand, nonviral methods are safe and do not require immunosuppression for successful gene delivery, but suffer from lower transfection efficiencies. DNA injection followed by application of electric fields (electroporation) has been more effective for introducing DNA than the use of simple DNA injection [87]. However, this method involves the concern that the electric pulse causes tissue damage. Recently, we reported that gene transfer using an ultra-fine needle [88], in addition to microbubbles enhanced transcutaneous sonoporation [87].

In vivo gene delivery seems to be a suitable gene therapy approach in tooth regeneration by stimulation of the third dentition.

6. Conclusion

We have a chance to access the formation of the third dentition in the mouth, because the timing of the appearance of the third dentition seems to be after birth. The identification of mutations in *RUNX2* causing an isolated dental phenotype in CCD and supernumerary tooth formation in the mouse model clearly demonstrated that it was possible to induce *de novo* tooth formation by the in situ repression or activation of a single candidate gene. These results support the idea that the *de novo* repression or activation of candidate genes such as *RUNX2* or *USAG-1* might be used to stimulate the third dentition in order to induce new tooth formation in the mouse (Figure 4). *In vivo* gene delivery seems to be a suitable gene therapy approach in tooth regeneration by stimulation of the third dentition.

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